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Effect of carotenoid volatiles on oviposition and feeding choice of T.ni and T.vaporariorum

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Abstract

Carotenoid cleavage dioxygenases (*CCDs*) are responsible for the cleavage of carotenoids into smaller compounds, including apocarotenoids. The volatile apocarotenoids produced have demonstrated a repellent and feeding deterrent effect with some insects. To understand the formation of apocarotenoids and the effect on insect oviposition and feeding preference, I investigated the role of *CCD* genes in plant-insect interactions by comparing four different transgenic genotypes that over-express *CCD*'s and the respective wild-type (WT) for two model plants. *CCD1* and *CCD4* genes were overexpressed in *Arabidopsis thaliana* (Arabidopsis) and *LeCCD1-1* and *LeCCD1-2* genes were overexpressed in *Solanum lycopersicum* (tomato), Oviposition choice bioassays with the cabbage looper moth (*Trichoplusia ni*) and greenhouse whitefly (*Trialeurodes vaporariorum*) showed a significantly greater oviposition of both insects on the transgenic plants in comparison to WT plants, whereas feeding assays with *T. ni* larvae indicated no preference toward CCD over-expressing plants. The findings suggest that manipulating the carotenoid-based volatile profile of plants could provide a novel strategy to attract pest insects away from the crops towards these trap plants. This would also contribute to a reduction in the dependence of chemical pesticides and reduce the associated negative environmental effects of their use.

Keywords

Trichoplusia ni, Arabidopsis thaliana, Lycopersicon esculentum, apocarotenoids, β-ionone, caryophyllene, olfaction, feeding, carotenoid cleavage dioxygenases, attractant, biosynthetic genes.

Statement of Co-Authorship

1. **S. Challa**, I.M. Scott, L.A. Cáceres, M.W. Sumarah, V. Grbic & A. Hannoufa. Oviposition and feeding responses of *Trichoplusia ni* Hubner (Lepidoptera: Noctuidae) to carotenoid-derived volatiles in *Arabidopsis thaliana.* Unpublished data (Chapter 2).

   The author was involved in the molecular characterization of the transgenic Arabidopsis plants, determination of the leaf carotenoid content, *In vivo* extraction of volatile organic compounds and biological assays with *Trichoplusia ni.* The manuscript was revised with I. Scott and M.W. Sumarah.

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   All experiments were designed and performed by the author. Transgenic tomato plants were provided by L. Tian. The manuscript was revised by I. Scott.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ABA	abscisic acid
bLYC	lycopene β-cyclase
bp	base pair
CCD	carotenoid cleavage dioxygenases
cDNA	complementary DNA
DMAP	dimethylallyl diphosphate
dNTP	deoxyribonucleotide triphosphate
eLYC	lycopene ε-cyclase
GC-MS	gas chromatography mass spectrometry
GGDP	geranyl geranyl diphosphate
GGDPS	geranyl geranyl diphosphate synthase
HPLC	high performance liquid chromatography
LUT1	ε-ring carotene hydroxylase
MEP	methylerythritol phosphate
MS	Murashige and Skoog
NaCl	sodium chloride
NaOH	sodium hydroxide
NCED	9-cis-epoxycarotenoid dioxygenase
PCR	polymerase chain reaction
PDS	phytoene desaturase
PSY	phytoene synthase
RNA	ribonucleic acid

RT-PCR	reverse transcription-polymerase chain reaction
SDS	sodium dodecyl sulfate
v/v	volume per volume
VDE	violaxanthin deepoxidase
w/v	weight per volume
WT	wild type
ZDS	ζ-carotene desaturase

CHAPTER 1: Introduction

Plants are sessile organisms exposed to many environmental factors such as heat, cold, drought, salinity, etc., that can hinder or reduce growth, development, reproduction and yield. Furthermore, plants co-exist and interact with other organisms such as other plants, bacteria, fungi, insects and animals that can cause similar negative effects. Despite the vulnerability of plants as sessile organisms to adverse biotic and abiotic conditions, plants dominate over much of the land surface. This is due to the evolved ability of plants to defend themselves by a combination of physical, chemical and developmental features. Physical characteristics such as thorns, spines and micro-needles reduce browsing by large herbivores (deer, moose, antelope, goats and giraffes) by slowing down the herbivores' feeding rate, and by wearing down their teeth (Belovsky et al., 1991; Gómez & Zamora, 2002). A few herbs, notable nettles, cover their epidermis with microscopic needles that inject acid into animal skin at a touch (Cooper & Owen-Smith, 1986). Some woody plants have bark that provides fire protection and some herbs have waxy cuticles that resist penetration by pathogens. Besides physical structures, various other modes of defense are employed by plants to protect themselves against the plethora of antagonists they face in nature. A high diversity of secondary metabolites have a predominant function in defense based on their toxic nature or repellence to herbivores and microbes or as an important means of communication between plants and insects. For example, the pyrethrins occurring in leaves and flowers of *Chrysanthemum* species act as strong insecticidal compounds to deter insects like beetles, wasps and moths (Turlings et al., 1995). Similarly, in gymnosperms, monoterpenes such as α-pinene, limonene and myrecene are toxic to numerous pests of conifer species (Turlings et al., 1995). Research has shown that volatiles produced by the breakdown of carotenoids also have an influence on insect behavior (Heath et al., 2013). For example,

overexpression of terpene synthases in Arabidopsis is known to attract enemies of herbivores under laboratory conditions (Schnee et al., 2006), while plants like maize that constitutively produce caryophyllene attract nematodes which are predators of corn rootworm *Diabrotica virgifera* (Degenhardt et al., 2009). Over time, plants have evolved physical and chemical systems to ward off, inhibit or kill their enemies, but modern agriculture is often a monoculture of one crop type that attracts multiple pests at a time. Therefore, farmers require an efficient strategy to protect crops from those organisms or risk losing the entire field. Cabbage looper moths *Trichoplusia ni* (Lepidoptera: Noctuidae) and greenhouse whiteflies *Trialeurodes vaporariorum* (Hemiptera: Aleyrodidae) are two significant agricultural pests that affect a wide range of vegetable crops (Li et al., 2006; Shelton et al., 1982; Van Lenteren & De Ponti, 1990; van Lenteren et al., 1996). Currently, chemical insecticides are considered the most reliable and effective means of controlling these insects on the field and in greenhouse conditions. However, too much reliance on chemical pesticides is becoming less acceptable due to a range of environmental and human health concerns, which provide major incentives for developing pest management strategies that are more sustainable and environmentally benign.

1.1 Pest control strategies

On a global scale, an estimated 20-40% of agricultural produce is lost to pathogens, insects and animals (Oerke, 2006). Damage due to insects alone and the costs associated with minimizing the loss are difficult to estimate accurately for they are dependent on a number of other factors such as environmental conditions, the plant species being cultivated and the technology being used (Oliveira et al., 2014). In addition to the economic losses caused by the damage to crops by different pests, measures taken to reduce the infestation by the pests also cause indirect economic losses. For example, in the United States alone, approximately 500 million kg of different

pesticides are applied annually at a cost of $10 billion, an amount that does not include the actual application costs. Pesticides are also linked to a wide range of human and environmental health hazards such as poisoning, endocrine disruption, water and soil contamination, loss of biodiversity and pesticide resistance (El-Bahnasawy et al., 2014). New research continues to uncover further negative impacts from pesticide use and indicates a very urgent need for the development of alternative strategies that enable protection of crops without causing a negative impact on human health and environment.

Of the many components of integrated pest management (IPM), scouting or monitoring of fields at regular intervals to assess whether the pest infestation remains below a damage threshold is perhaps the simplest and cheapest strategy to determine when spraying a pesticide is required. Cultural control is another approach by which pest control is achieved and includes techniques such as crop rotation, tillage, use of trap crops and companion planting (Fereres, 2000). Biological control agents such as parasitoids, predators and pathogens have been successfully used as an economical alternative to chemical pest control in some agricultural systems such as orchards, vineyards and greenhouses (Greathead, 1995). However, this strategy like others also has certain limitations. For example, most biological control agents are host-specific as each agent is often active against a single pest species. This requires the use of many different agents to control a broad spectrum of pests found in fields. The rate of action of these agents is also relatively slow in comparison to the alternative quick fix - chemical pesticides. Furthermore, the performance of biocontrol is subject to environmental factors that are often site- and host biotype-specific. In contrast use of chemical pesticides can be more generally applied, and became popular beginning in the mid-20th century with the invention of synthetic pesticides such as dichlorodiphenyltrichloroethane (DDT), an organochloride insecticide. The long term

negative effects of DDT and other synthetic pesticides along with increasing consumer awareness regarding food quality and environmental concerns are now leading to a renewed interest in natural pest control strategies. Controversies involving conventional agriculture such as mad cow disease and genetically modified organisms, has been heightened consumers interest in organic products which gives another reason for developing environmentally friendly farming strategies (Forge, 2001).

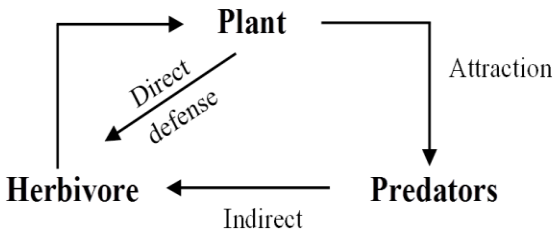
Utilizing the plant's self-defense mechanisms is one approach that is gaining increased attention (Birkett & Pickett, 2014; Pickett et al., 2014). This approach is inexpensive, compatible with insecticide use and is density-independent contrary to the use of biological control agents.

1.2 Plant defense mechanisms against insect herbivores

Although lacking an immune system akin to humans, plants have developed a large number of structural, chemical and protein-based defenses designed to detect invading organisms and stop them before they are able to cause extensive damage. These defenses are broadly classified into direct and indirect defenses (Figure 1.1). Both (direct and indirect) defense mechanisms may be present constitutively or induced after damage by the herbivores or disease.

Traditional plant characteristics that directly affect herbivores involve both physical and chemical defenses. Physical defenses include the many structural defenses of plants such as spines, thorns or trichomes, small hairs on the leaf surface that impede herbivore movement (Fernandes, 1994). Chemical defenses include a wide range of defense metabolites, anti-digestive compounds, anti- anti-nutritive proteins or peptides that negatively influence herbivore physiology (Howe & Jander, 2008). Thousands of plant secondary metabolites that function as defense chemicals have been identified and grouped into major classes including nitrogen-

A)



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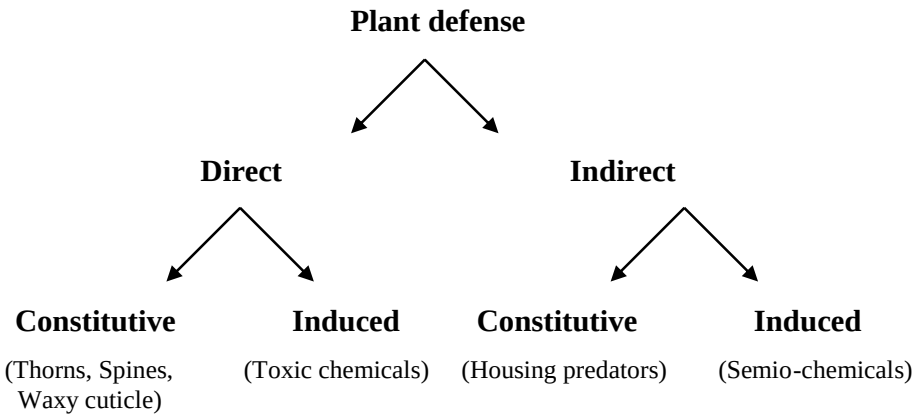


Figure 1.1 Defense strategies in plants. A) Tri-trophic interaction between plants, herbivores and predators. B) Classification of the different defense strategies developed by plants

containing metabolites like alkaloids and glucosinolates, or phenolics like phenylpropanoids, flavonoids and terpenoids.

The indirect defenses plants may employ include the signaling of natural enemies of herbivores (such as predators or parasitoids) that act as "bodyguards" (Sabelis et al., 1999) and provide protection to the plant by attacking the herbivores. Plants also provide floral or extra floral nectar that carnivorous arthropods feed on and the production of these nutrient sources can be induced by herbivory (Karban & Baldwin, 2007). It is well known that elicitors in herbivore oral secretions can induce an anti-herbivore response in plants (McCloud & Baldwin, 1997). β-glucosidase is one of the best known examples of an herbivore elicitor. Jasmonate metabolites also play key roles in direct defense responses as the concentration of jasmonates rapidly increases in the early stages of herbivore attack (Berger et al., 1995; Halitschke et al., 2003; Paschold et al., 2007). Finally, plants may lure or deter carnivorous arthropods with plant volatiles produced in response to herbivore attack (Knight et al., 2011; Wei et al., 2007). This has been observed through the repellence of aphids by wheat seedlings infested by the high density of aphids (Quiroz et al., 1997). Results from the study showed that aphids (*Rhopalosiphum padi*; Hemiptera: Aphididae) in an olfactometer were attracted towards volatiles from an undamaged wheat seedling and were repelled by a wheat seedling with a high aphid density by the feeding the crucifer pest *Pieris rapae* caterpillars that leads to volatiles released to attract the parasitoid wasp, *Cotesia rubecula*, predators of the *P. rapae* caterpillars (Van Poecke et al., 2001).

Plant secondary metabolites as mediators of defense related ecological interactions (Hartmann, 2008) are also important in the role of deterring herbivores (Frenkel, 1959). Plant volatile organic compounds (VOCs) in particular, have been at the center of intensive studies of plant-

insect interactions (Tumlinson et al., 1999). Improvements in analytical techniques, molecular and biochemical methods and the development of static and dynamic techniques for headspace collection of volatiles in combination with gas chromatography-mass spectrometry (GC-MS) analysis are significant reasons for the advancement in this field of research (Bicchi & Maffei, 2012).

1.3 Role of volatiles in plant-insect interactions

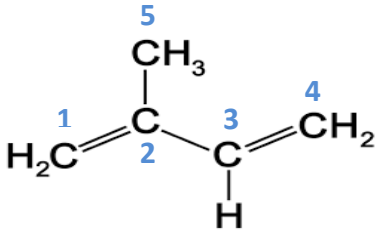
The emission of volatile organic compounds (VOCs) from plant tissues has been recognized as an important component in the interaction between plants and insects for many years, both in the attraction of pollinators and the deterrence of herbivores. VOCs have the potential to shape aboveground arthropod communities as well as belowground microorganism and macroorganism communities (Bezemer & van Dam, 2005). Belowground VOCs released due to insect attack are known to induce aboveground resistance (Erb et al., 2009). Aboveground, the volatiles emitted by plants play a vital role in both direct and indirect defense strategies. As a direct defense, species-specific volatiles, for example, monoterpenes in pine, can have a repellent or toxic effect (Litvak & Monson, 1998). Additionally, there is evidence for oviposition deterrence by induced volatiles, e.g., from herbivore damaged tobacco plants, to deter oviposition by lepidopteran herbivores (De Moraes et al., 2001; Kessler et al., 2008).

Approximately 3000 plant volatile compounds have been identified to date. These compounds include terpenoids, phenylpropanoids/benzenoids, fatty acid derivatives and amino acid derivatives (Dudareva & Pichersky, 2000). Studies have shown that the composition of the volatile compounds emitted depends on factors including the plant and insect species (Das et al., 2013). Among the different types of VOCs, terpenoids represent the largest class and are well

known to act as toxins, feeding deterrents or oviposition deterrents to a large number of insects (Wei et al., 2004). These compounds are derived from the isoprenoid pathway as shown in previous studies that successfully engineered this pathway by manipulating genes and the gene products of this pathway. In a recent study, Wei et al. (2011) showed that engineering the isoprenoid pathway led to an increase in β-ionone, a terpenoid-derived volatile, which had negative effects on crucifer flea beetle *Phyllotreta cruciferae* Goeze (Coleoptera: Chrysomelidae) feeding. Considering the importance of terpenoids, this thesis has focused on the carotenoid-derived volatiles and their interaction with insects.

1.4 Carotenoids and apocarotenoids

Among the various volatile compounds that are involved in plant resistance, isoprenoids also known as terpenoids, are the largest class of secondary metabolites that are actively involved in plant defense against herbivorous insects (Deka & Bora, 2014; Rodríguez et al., 2014; Theis et al., 2014). Terpenoids are terpenes, or simple hydrocarbon molecule that has been modified by the addition of oxygen or removal or repositioning of a methyl group. The basic unit of terpene or terpenoid consists of isoprene, a simple five-carbon molecule, which is the building block of most plant metabolites including hormones, sterols and carotenoids (Huang et al., 2012; Langenheim, 1994). A single isoprene unit (Figure 1.2) represents the most basic class of terpenes, the hemiterpenes. An isoprene unit bonded with a second isoprene is the defining characteristic of a terpene, also referred to as a monoterpene (C10). Sesquiterpenes contain three isoprene units (C15), while diterpenes (C20) and triterpenes (C30) contain two and three terpene units, respectively. Tetraterpenes consist of four terpene units and the most prevalent tetraterpenes are the carotenoid accessory pigments which perform essential functions in



Isoprene

Figure 1.2 Basic five carbon unit, isoprene

photosynthesis. Polyterpenes are those terpenes that contain more than four terpene units (i.e., more than eight isoprene units). The vast array of terpenes have many applications in the pharmaceutical and food industry and in agriculture (Aharoni et al., 2005). Terpenoid-derived volatiles have been documented to act as toxins, feeding deterrents, or oviposition deterrents to various insects. Volatile essences of flowers are comprised of monoterpenes. These compounds have been exploited by man in the manufacture of flavours and perfumes. Similarly, sesquiterpenes are found in essential oils and are also known to discourage herbivory (Vickers et al., 2014). Terpenoids are also a great source of pharmacologically important metabolites such as taxol, an anticancer agent. Due to an array of important functions, investigations of terpenoids saw an increase at the turn of the 20th century (Locher et al., 2013).

Carotenoids form an important group of natural terpenoids. They are a class of isoprenoid pigments, which provide nutritional and functional value. In humans, carotenoids have been implicated in preventing various eye and cardiovascular diseases. Carotenoids are well known for their antioxidant qualities and/or regulators of the immune system. Carotenoids are also critical components of the photosynthetic machinery, and play a role in protecting the plant from photooxidative damage (Howitt & Pogson, 2006). In this context, there is considerable interest in the manipulation of carotenoid content and composition in plants to improve the agronomic and nutritional value for human and animal consumption. Furthermore, the suite of defense-related carotenoid-derived volatiles gives additional reasons for targeting the carotenoid biosynthetic pathway for genetic engineering (Schmidt-Dannert et al., 2000). Carotenoids are in constant turnover; i.e., biosynthesis and catabolism, and oxidative cleavage of carotenoids produces apocarotenoids (Wahlberg & Eklund, 1998). Apocarotenoids include biologically active compounds such as the plant hormones abscisic acid (ABA) and strigolactone (SL), as well as

flavor and fragrance compounds (Mendes-Pinto, 2009). Some of the commonly known volatile apocarotenoids include β-ionone, β-cyclocitral, theasporone, β-damescenone, and α-damescenone. Apocarotenoids are generated when double bonds in the carotenoid backbone are cleaved by molecular oxygen forming an aldehyde and ketone from each substrate at the site of cleavage. Carotenoids can be cleaved at any of their double bonds resulting in a diverse set of apocarotenoids (Vogel et al., 2008). These apocarotenoids can be both volatile and non-volatile. This thesis focuses on the volatile apocarotenoids derived from carotenoids. The proven potential of these volatiles to influence insect behavior led to this investigation into the role of volatile apocarotenoids as repellents or attractants to insect oviposition and feeding choices (Cáceres, 2015; Lakshminarayan, 2013; Wei et al., 2011).

The carotenoid biosynthesis pathway responsible for the production of apocarotenoids has been well investigated and has led to many successful genetic engineering attempts (Giuliano et al., 2008; Goo et al., 2015). Here, I briefly summarize the pathway (Figure 1.3) to help better understand the role of CCD enzymes and their interactions with other elements. The central metabolite or the building block for all isoprenoid compounds is the 5-carbon isopentyl pyrophosphate (IPP). Various isoprenoids with 5, 10, 15, 20 and more carbons in their skeletal structure are formed by a molecular assembly process involving very few reaction steps (Misawa et al., 1995). For instance, carotenoids containing 40 carbons are assembled from two molecules of a C20 compound, geranyl geranyl pyrophosphate (GGPP). GGPP itself is formed from four units of IPP. Geranyl-geranyl diphosphate synthase (GGDPS) catalyses the condensation of three molecules of IPP with one molecule of dimethyl diphosphate (DMAPP) to produce a 20-carbon molecule, GGDP, which is the precursor of the carotenoid biosynthesis pathway. The first committed step in the carotenoid biosynthesis pathway is the condensation of two GGDP

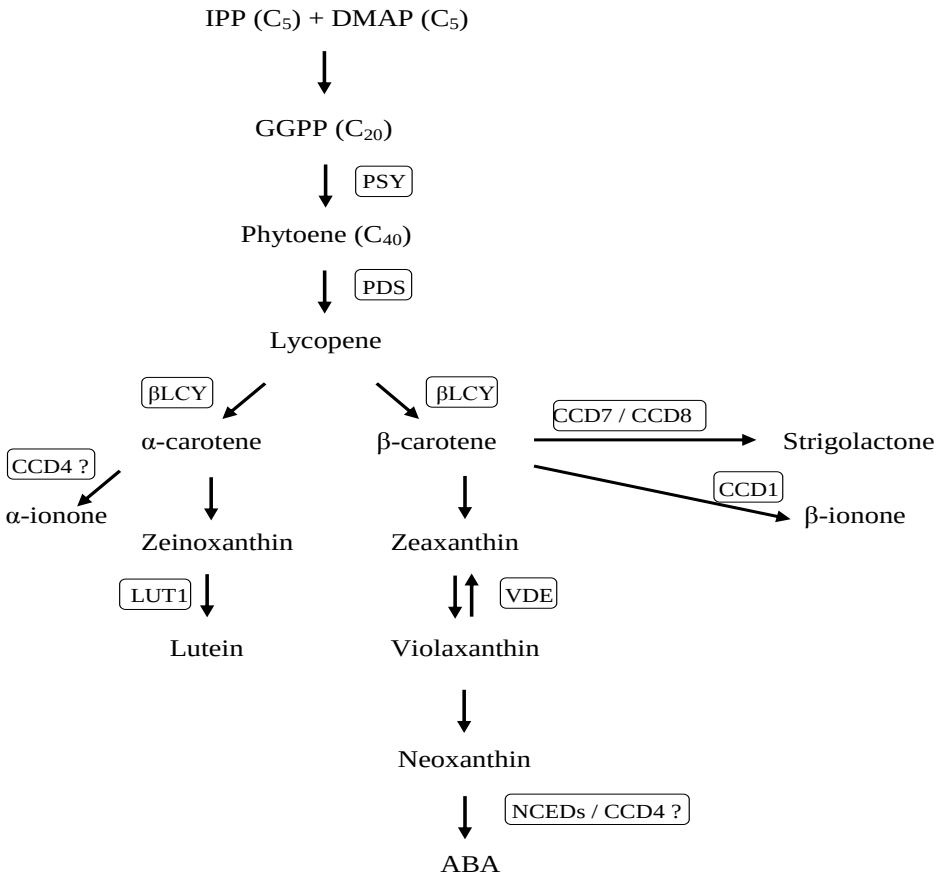


Figure 1.3 Carotenoid biosynthesis and turnover pathway in Arabidopsis. The arrows indicate biosynthetic steps. GGPP, geranylgeranyl phosphate; NCED are genes encoding 9-cis-epoxycarotenoid dioxygenases which are involved in ABA biosynthesis. *CCD1, 4, 7* and *8* are genes encoding carotenoid cleavage dioxygenases 1, 4, 7 and 8 respectively.

molecules by phytoene synthase (PSY) to produce a 40-carbon molecule, phytoene, considered a rate-limiting step (Lu & Li, 2008). The next step involves the desaturation of phytoene into red colored lycopene by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS). Lycopene is the critical branching point in the pathway (Cazzonelli & Pogson, 2010). It is cyclized to yield either α-carotene by lycopene ε-cyclase (eLYC) and lycopene β-cyclase (bLYC) or β-carotene by bLYC alone. α-carotene and β-carotene are hydroxylated to produce lutein and zeaxanthin, respectively. These hydroxylation reactions are catalyzed by the β-ring carotene hydroxylase and the ε-ring carotene hydroxylase (LUT1) (Tian et al., 2004). Lutein is one of the most abundant carotenoids, and is present in the leaf tissues of most plants. Epoxidation of zeaxanthin by zeaxanthin epoxidase (ZEP) produces violaxanthin. This reaction is reversed by violaxanthin deepoxidase (VDE) to give rise to the xanthophyll cycle, which helps plants acclimatize to high light stress. Violaxanthin is further converted to neoxanthin by neoxanthin synthase (NSY). The formation of neoxanthin represents the last step in the carotenoid biosynthesis pathway (Lu & Li, 2008). The end products of the pathway can be catabolized to produce apocarotenoids. The carotenoid cleavage dioxygenase (CCD) enzymes target various non-specific carotenoids in the pathway, whereas the 9-cis-epoxycarotenoid dioxygenases (NCEDs) are predominantly responsible for cleaving violaxanthin and neoxanthin to produce xanthoxin, the direct substrate for ABA synthesis. The enzyme ABA2 uses xanthoxin as a substrate in the ABA conversion step. It is obvious that the carotenoid biosynthetic pathway is amenable to genetic engineering, and thus natural pest management might be achieved.

1.5 Model plant systems

1.5.1 *Arabidopsis thaliana* as the model plant for studying plant-insect interactions

Arabidopsis thaliana (Arabidopsis) is a largely selfing, annual plant native to Germany, but is widely found throughout Europe, Asia and North America (Koornneef & Meinke, 2010). Over the last 20 years, Arabidopsis has become universally recognized as a model for plant research. The reasons for this include its small size, short life cycle (approximately 3 months from seed to seed), easy and inexpensive maintenance and large number of seeds (Meinke et al., 1998). It is also the first plant to have an extensive knowledge base which includes full genome sequence, transcriptome, proteome and metabolome datasets, information on protein interactions, hundreds of genotyped accessions and germplasm banks. These factors and the ability to transform Arabidopsis have made it one of the favourite plant model systems for molecular genetic studies. Arabidopsis has also provided valuable information on plant-insect interactions, including those involving insects in the orders Coleoptera, Diptera, Hemiptera, Lepidoptera and Thysanoptera. Many research groups have successfully utilized Arabidopsis to gain important insights about genes and mechanisms that contribute to plant resistance; for example the role of jasmonic acid and how alterations in its level affects the plant susceptibility to insect herbivores (Anderson et al., 2004; Birkett et al., 2000).

1.5.2 *Solanum lycopersicum* as the model plant for studying plant-insect interactions

Plant scientists consider *Arabidopsis thaliana* as an excellent model plant for genome manipulation. Although much information on plant-microbe interactions have been accumulated using this model plant, additional models are required for a comprehensive evaluation of plant-pathogen interactions. One reason is the small number of pathogens associated with Arabidopsis,

including an underrepresented group of pathogens called Ascomycetes among the Arabidopsis pathogens (Arie et al., 2007). In contrast, solanaceous plants which include many agriculturally important crops (tomato, potato, tobacco, pepper, egg-plant) as well as ornamental and medicinal plants (Capsicum, *Atropa belladonna*) have provided excellent alternative model systems to study plant-pathogen interactions (Emmanuel & Levy, 2002; Meissner et al., 1997).

Among the Solanaceous plants, tomato (*Solanum lycopersicum*, formerly *Lycopersicon esculentum*) is one of the most popular vegetables worldwide. However, its cultivation is limited due to susceptibility to a range of pathogens including fungi, bacteria, viruses, various nematodes and insects. This diversity of pathogens and insects makes tomato a favorable model for studying plant-insect interactions. Additional reasons for using tomato as a model plant for most studies are: a) tomato is one of the smallest diploid genomes among the Solanaceae species for which homozygous inbred lines are available and b) Solanaceae plants show very high conservation thus the tomato genome will enable comparative genomics among the different Solanaceae species and improvement of desired traits by refined molecular breeding strategies, enabled in part by the use of stable plant transformations.

In order to deepen our understanding of the ecological interaction of these model plants and herbivores, it is important to choose an insect model for the analysis of insect feeding and oviposition behavior.

1.6 Model insect systems

1.6.1 *Trichoplusia ni* (Cabbage looper moth)

Cabbage loopers are chewing insects that feed by night on a number of important crop plants such as broccoli, cabbage, cauliflower, Chinese broccoli, Chinese cabbage, daikon, flowering

white cabbage, lettuce, beet, peas, celery, tomato and certain ornamental plants. Geographically, it is widespread (Vail et al., 1971) and has been found in environments ranging from North America to the UK, Turkey, and eastward to India and Japan (Caster, 1980; Brown, 1982; Kirby, 1982; Nasu et al., 2003). Alfalfa loopers and cabbage loopers are two common types of looper worms that infect the crop plants. Of the two species, cabbage looper affects a larger variety of crops and is a serious pest in field and greenhouse conditions. The focus of this project therefore is the cabbage looper moth. The cabbage loopers lack walking appendages or legs in the middle of the body and move forward by drawing the rear end up to the front end and the straightening. Three pairs of legs are present on the thorax and three pairs of prolegs are on the abdomen (one pair on segment five and six and one pair on the terminal segment).The movement, therefore, resembles a looping motion, similar to that of an inchworm. Hence, their common name "looper". The young larvae are voracious feeders of green plant tissue and leave ragged holes in the leaves, mainly between the veins. The older larvae cause more extensive damage and are capable of completely defoliating plants. The excreta of the loopers is dark green in colour and is referred to as frass. When the looper numbers are high, damage may be enough to stunt growth or prevent head formation in cabbage and similar crops. Hence, they are a serious agricultural pest (Shropshire, 1935).

Description and life history

Older loopers or caterpillars have a smooth light green body, usually with a white stripe down each side and reach a length of 1 ¼ inch (3.2 cm). Younger larvae tend to be paler. Adult moths are greyish brown, but can be recognized by a characteristic white or silver "Y" or a "figurative 8" mark on each forewing (Creighton, 1980).

Adult cabbage looper moths migrate to northern areas in spring or summer. Moths deposit eggs on host plants, usually singly. Cabbage looper eggs are round, very pale green to white, and found generally on the lower surface of the leaves. The eggs hatch in 2-10 days, dependent on temperature (Gaikwad et al., 1980). The larvae pass through five instars based on head capsule size. Maximum weight gain (up to 68%) occurs during the fifth instar. Early instar larvae feed on the lower surfaces of leaves producing small holes that do not break through the upper surface of the leaf. Larger caterpillars do more extensive damage to the entire plant. The caterpillars feed on plants for three-four weeks. Mature larvae pupate on the undersides of foliage or in the soil. Pre-pupation is indicated by a lighter, uniform body colour of the larvae and cocoon-spinning, which lasts for 1 day. Pupation lasts for about 8 days. The adult emerges in approximately 3 days and typically survive 6 to 9 days. Multiple generations of usually three to four occur during the growing season (Shorey, 1962; Henneberry, 1966).

1.6.2 *Trialeurodes vaporariorum* (Greenhouse whitefly)

Whiteflies are tiny sap-sucking insects and are globally distributed as agricultural pests of both greenhouse and field crops. Although > 1,500 species of whiteflies exist, the primary pest species of whitefly is the greenhouse whitefly, *Trialeurodes vaporariorum* (Hemiptera: Aleyrodidae) and the sweet potato whitefly, *Bemisia tabaci* Gennadius (Hemiptera: Aleyrodidae). Of the two species, *T. vaporariorum* is the primary pest of greenhouse crops and hence is the focus of this project.

Description and life history

Their common name, whitefly, is due to the presence of white wax and lipid particles that are present over the body and wings of most adult species (Byrne & Hadley, 1988; Buckneret al.

1994). *T. vaporariorum* are polyphagous herbivores that reduce crop yields by extracting water, carbohydrates and amino acids from plant phloem (Lloyd, 1922). As phloem-feeding whiteflies excrete sticky honeydew that can cover fruit and foliage of crops. Honeydew fosters the growth of sooty mold (*Cladosporium*) on plants and reduces plant photosynthesis (Lloyd, 1922; Hoddle et al., 1998; Smith et al., 2001).

Adult whiteflies are moth-like with yellowish bodies and wings covered with white, waxy powder. They are about 1/16 of an inch in length. *T. vaporariorum* species can be identified from the rest of the whitefly species by the shape of its wings. These species hold their wings flat, giving them a triangular appearance from above. Whiteflies are "true bugs" (Hemipterans) and undergo hemimetabolous development that includes three distinct stages: egg, nymph and imago (adult). The *T. vaporariorum* life cycle consists of six stages including the egg, the crawler (1^{st} nymphal instar), two sessile nymph instars (2^{nd} and 3^{rd} instar), the pupa (which is further divided into three substages: the 4^{th} instar, the prepupa and the pupa) and the adult or imago (Gill, 1990). During oviposition, eggs are often laid on the undersides of plant leaves in a circular fashion and the female will continue to feed on plant sap while rotating its rostrum to deposit eggs. Eggs are whitish to light beige in colour but darken to a dark blue or black colour before hatching. Eggs are secured to the plant by a short stalk, called pedicel (Gill, 1990). The egg pedicel is either inserted into a slit in the leaf surface (made by the ovipositor) or into a stomato opening. In addition to securing the egg to the plant, the pedicle is thought to function as a water source for eggs (Byrne et al., 1990).

When the eggs hatch, the subsequent larvae (called the first instar, or crawler) move a short distance from the site of egg hatching in search for feeding sites (Byrne and Bellows, 1991; Martin et al., 2000). The crawler is the only immature form that is mobile with functional

walking legs and antennae. The duration and distance of crawler movement depends on the crawler's ability to locate acceptable feeding sites.

1.7 Scope of the research project

Carotenoid cleavage dioxygenases (CCD) and 9-cis-epoxycarotenoid dioxygenase (NCED) were reported to cleave a broad range of carotenoids at specific double bonds, to generate apocarotenoids (Auldridge et al., 2006; Ohmiya, 2009) which in turn play a role in plant-insect interactions (Heath et al., 2012; Rubio et al., 2008; Wei et al., 2011). Since *CCD*s (*NCED* and *CCD*) are highly conserved and originate by duplication and divergence of a common protein, I hypothesized that these effects would also be observed in the Arabidopsis-cabbage looper moth system, tomato-greenhouse whitefly system and tomato-cabbage looper moth system. To investigate my hypothesis, transgenic Arabidopsis and tomato plants overexpressing *CCD1*, *CCD4* and *LeCCD1-1* and *LeCCD1-2* genes were first generated and the carotenoid levels in each genotype were determined. The volatile profile of the different genotypes was then determined using gas chromatography - mass spectrometry (GC-MS). Finally, the effects of the carotenoid-derived volatiles on insect oviposition and feeding choice were investigated.

The effects of carotenoid-derived volatiles on insect feeding choice was observed and recorded previously (Lakshminarayan, 2013; Wei et al., 2011). Hence, I also expected a decrease in the percentage of leaf area consumed by cabbage looper larvae. The feeding choice was determined by scoring the leaf damage using a recognized method (Hallett et al., 2005). Finally, I predict that the deterrence of feeding and oviposition due to the volatiles emitted by the transgenic plants would provide a safe environmentally friendly alternative for pest management.

1.8 Hypothesis and objectives

I hypothesize that:

a) Over-expression of *CCD* genes will result in enhanced emission of volatile apocarotenoids

b) Higher apocarotenoid levels will deter cabbage looper moth and greenhouse whitefly oviposition and feeding

The objectives of this study are:

- To generate *CCD* overexpression Arabidopsis and tomato genotypes
- To investigate the effects of overexpressing carotenoid catabolism genes (*CCD1, CCD4, LeCCD1-1* and *LeCCD1-2*) on the carotenoid levels
- To analyze the volatile profile of transgenic Arabidopsis and tomato and to identify the different volatile compounds generated
- To assess the biological effects of VOCs produced *in vivo* on insect feeding and oviposition choice

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Chapter 2: Oviposition and feeding responses of *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) to carotenoid-derived volatiles in *Arabidopsis thaliana*

2.1 Introduction

Plants release a variety of volatile organic compounds that play critical roles in interactions with the environment (Cascone et al., 2015; De Alfonso et al., 2014; Dudareva & Negre, 2005). Insect herbivores exploit these volatiles to locate their host plants for feeding and oviposition. For example, the red-legged earth mite *Halotydeus destructor* Tucker (Acari: Penthaleidae) fed less on *Trifolium glanduliferum* (Fabales: Fabaceae) that had high levels of β-ionone and other terpenes (Wang et al., 2005). Similarly, methyl salicylate inhibited feeding and egg-laying activity by western flower thrips *Frankliniella occidentalis* (Thysenoptera: Thripidae) when applied to the leaf surface of bean and cucumber (Koschier et al., 2007). However, modern breeding strategies and domestication of crops are leading to growth-defense trade-offs in the plants (Dudareva et al., 2013). Manipulating the plant genome for improved growth or yield negatively effects the plant secondary metabolism, especially VOC production (Tamiru, 2012). These studies suggest that there is a possibility of manipulating plant-insect interaction and promoting pest resistance (Akhtar et al., 2012) by engineering metabolites of the plant volatile spectrum (Dudareva et al., 2013; Vickers et al., 2014).

Plant volatiles are products of diverse metabolic pathways, but most are derived from the isoprenoid or terpenoid pathways (De Moraes et al., 2001; Wei et al., 2004). Terpenoids, the predominant class of volatiles are derived from five-carbon isoprene units assembled and modified in many ways (Langenheim, 1994). Carotenoids (C40 isoprenoids) (Lu & Li, 2008) are one of the most studied classes of terpenoids that play critical roles not only in plant defense, but

in plant growth and development, in addition to their many economic and health benefits (Fernández-García et al., 2012). They are precursors of vitamin A, and some carotenoids are also used as food colorants in the food and cosmetics industries (Beatty et al., 2004; Umeno et al., 2005). Carotenoids are multifunctional compounds that serve as structural components of the light harvesting complexes, and are critical components of the photosynthetic machinery and scavengers of singlet oxygen, protecting the plant from photo oxidative damage (Howitt & Pogson, 2006). They provide the yellow, orange, and red colors to fruits and flowers (Baroli et al., 2000).

Carotenoids are in constant turnover; i.e., biosynthesis and catabolism, and specific enzymatic cleavage of carotenoids produces various types of biologically active compounds such as vitamins, phytohormones, aroma compounds and apocarotenoid pigments (Ebeler & Winterhalter, 2013) Apocarotenoids are multifaceted compounds including biologically active compounds such as abscisic acid (ABA), strigolactones (SL), aroma and flavor compounds, regulatory compounds and compounds with yet unknown functions (McCarty, 1995; Walter et al., 2010). ABA plays a key role in seed development and in plant response to environmental stresses (Nambara & Marion-Poll, 2005). Strigolactones are signaling compounds that regulate shoot branching and promote symbiotic interactions between plants and soil microbes (Chevalier et al., 2014). In addition to these bioactive compounds, carotenoid catabolism produces many volatile apocarotenoids that not only provide unique flavor and aroma to fruits and flowers of many plant species (Mendes-Pinto, 2009), but are also associated with certain defense functions. Examples of commonly known volatile apocarotenoids include β-ionone, β-cyclocitral, theasporone, β-damescenone, and α-damescenone, among which, β-ionone and β-cyclocitral have documented effects on insect feeding and oviposition. Previous studies demonstrated that

β-ionone attracts beetles, *Anomala transvaalensis* (Coleoptera: Rutelidae) (Donaldson et al., 1990) and α- and β-ionol attract Solanum fruit fly *Batrocera latifrons* Hendel (Dipera: Tephritidae; (Flath et al., 1994). The model plant *Arabidopsis thaliana* (Arabidopsis) also produces a suite of terpenes that have been shown to have a defense function against many herbivores (Kappers et al., 2005; Sun et al., 2010). However, little is known about the enzymes responsible for the synthesis of these apocarotenoids. Carotenoid cleavage dioxygenases are known to be involved in the formation of diverse terpenoid compounds but the specific activity of each enzyme is still not fully understood.

In Arabidopsis, the gene family that encodes carotenoid catabolism enzymes comprises at least nine members, five of which code for the 9-cis-epoxycarotenoid dioxygenases (*NCED*: *NCED2, NCED3*, *NCED5*, *NCED6* and *NCED9*). The remaining four code for the carotenoid cleavage dioxygenases (*CCD*: *CCD1*, *CCD4, CCD7* and *CCD8*) (Harrison & Bugg, 2014). CCD and NCED enzymes differ on the basis of their preferred substrate and presumed mechanism of catalysis (Auldridge et al., 2006b). 9-cis-epoxycarotenoid dioxygenases are potentially involved in the generation of ABA via asymmetrical cleavage at 11 and 12 (11', 12') double bonds of neoxanthin and/or violaxanthin (Vogel et al., 2008). CCD enzymes on the other hand, cleave the 9, 10 (9', 10') double bonds of multiple carotenoid substrates to produce dialdehydes and ketones (Floss & Walter, 2009). It was demonstrated that *CCD1* cleaves the 9, 10 (9', 10') double bonds of multiple carotenoid substrates to produce a C14 dialdehyde and two C12 cyclohexane derivatives (Schmidt et al., 2006).

Knowledge of the volatile compounds and the mechanisms by which both plants and insects produce and react, respectively, to each other's signals is essential for a better understanding of plant-insect relationships in the context of the plants being attractive or disagreeable to the

insects for feeding and oviposition. It is difficult to isolate the effect of individual floral volatile components on insect behavior by studying naturally occurring variation. These difficulties are both qualitative and quantitative in nature and are mainly associated with sampling techniques (D'Alessandro & Turlings, 2006). Laboratory experiments, on the other hand, may allow only limited inference on natural populations because environmental conditions, as well as herbivores or pests, can strongly influence floral traits, particularly headspace volatiles. Approaches that allow headspace volatile profile manipulation under field conditions include the use of genetic technologies such as enhancing the expression of biosynthetic pathways by *Agrobacterium*-mediated transformation techniques.

The objectives of this study were to investigate 1) whether transgenic Arabidopsis plants that overexpress individual *CCD* genes have altered carotenoid levels and headspace carotenoid-derived volatiles and 2) whether the volatiles affect oviposition and feeding preference by cabbage looper *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae). The carotenoid and volatile profiles for each type of transgenic plant were measured by chromatography and correlated with the effect on cabbage looper in order to identify the compounds responsible for larval feeding and moth oviposition preference.

2.2 Materials and methods

2.2.1 Insect rearing

A laboratory population of *T. ni* originated from a colony at the Forest Pest Management Centre, Natural Resources Canada, Sault St. Marie, ON. After transfer to the Southern Crop Protection and Food Research Centre (SCPFRC), Agriculture and Agri-Food Canada (AAFC), London, ON, the *T. ni* were reared at 28 °C under a L16:D8 photoperiod on a meridic diet (Chippendale,

1965). Newly enclosed adults were sorted by sex and kept in separate plexiglass cages in a growth chamber set at 24 °C and 55% relative humidity (rh), L16:D8 photoperiod. Insects were kept at 4 °C for thirty min prior to being used in assays, to restrict movement and allow ease of handling.

2.2.2 Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used in transformation assays and all other aspects of this work due to a) its close relation to numerous agriculturally important Brassica crop species such as broccoli, mustard and cabbage and b) its self-fertilizing and short-life cycle. To evaluate the effects of different genotypes on *T. ni* oviposition preferences, three genotypes of Arabidopsis were selected: the Col-0 wild-type (WT) and two genetically manipulated transformants, the transgenic genotype 35S::CCD1 (CCD1) and 35S::CCD4 (CCD4) with three lines from each genotype (L1, L2 and L3). WT Arabidopsis seeds were obtained from the Arabidopsis Biological Resource Centre (Ohio State University, Columbus, OH), and the over-expression lines CCD1 and CCD4 were generated using *CCD* transgene expression cassettes. Plants were grown in pots containing ProMix BX potting soil (premier Horticulture, Quackertown, PA, USA) or on sterile MS (Murashige and Skoog Basal salt mixture) (Phyto Technology Laboratories, USA) media plates containing hygromycin B at a concentration of 25 μg/ml. All seeds were cold-stratified in the dark at 4 °C for two-day- and two-week-old seedlings from the MS media plates were transferred to the pots, and then pots were moved into growth chambers with a L16:D8 photoperiod [100 to 120 μmol/m^2/s] and 70% rh.

2.2.3 Cloning and transformation of transgenic lines

The *CCD* transgene expression cassettes were constructed by using full length cDNA of *CCD1* and *CCD4* genes that were amplified by PCR using primers CCD1-For and CCD1-Rev for *CCD1* and CCD4-For and CCD4-Rev for *CCD4* (Table 2.1). The fragments were cloned into the Gateway pENTRD vector (the entry vector; Life Technologies) and the resulting constructs were transferred to *Agrobacterium tumefaciens* cells (GV3101 strain; containing rifampicin and gentamycin resistance) using electroporation. The Agrobacterium strain was then used to transform Arabidopsis by the floral dip method (Clough & Bent, 1998). Transgenic plants were screened for the presence of the transgene by PCR using the forward primer 35SF3-For and the gene-specific reverse primers (CCD1-Rev or CCD4-Rev) (Table 2.1), and seed segregation analysis (based on resistance to hygromycin) was performed to select homozygous lines for further analysis and insect oviposition trials.

2.2.4 RNA isolation and gene expression analysis

The differential expression of the transgenes (*CCD* genes) and other carotenogenic genes (*ε-ring carotene hydroxylase (LUT1), β-carotene hydroxylase (BCH1), violaxanthin de-epoxidase (VDE), zeaxanthin epoxidase (ZEP), ζ-carotene desaturase (ZDS), lycopene β-cyclase (bLYC), ABA Deficient 2 (ABA2), phytoene synthase (PSY)* and *phytoene desaturase (PDS)*) was quantified by qRT-PCR using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Canada) (Bustin et al., 2009; Taylor et al., 2010). Total RNA was isolated from four week old rosette leaves using RNeasy Plant Mini Kit (Qiagen) followed by TURBO DNA-free Kit (Life Technologies, Burlington, ON) treatment to minimize genomic DNA contamination. The reverse

Table 2.1 List of primer sequences used for PCR and qRT-PCR analysis in this study

Gene	Primer name	Sequence (5′- 3′)	Primer Use
	35SF3	CAATCCCACTATCCTTCGCAAGACCC	Specific for 35S promoter in pMDC-32
CCD4	CCD4-Rev	TAAAGCTTATTAAGGTCACTTTCCTTGACAA	Gene specific sequencing
	CCD4-Int-For	TCACGCCATAAAAATCCACAACG	Gene specific sequencing
	CCD4-Int-Rev	CGTGAATGATATTGAATCCAGGAACTTC	Gene specific sequencing
	qRT-*CCD*4-For	CGGAGGCGGAGGAGGATGATG	Gene specific for q-PCR
	qRT-*CCD*4-Rev	CGGCGGCGACGATTTCAAG	Gene specific for q-PCR
CCD1	CCD1-Rev	GAAATCCATGGACGGGAGATCC	Gene specific sequencing
	CCD1-Int-For	TCAAAGTTTTGGAAGATGGAGACCTGC	Gene specific sequencing
	CCD1-Int-Rev	GCGTTGTGGAAAATAAAGCAGTTG	Gene specific sequencing
	qRT-CCD1-For	CGGAGGCGGAGGAGGATGATG	Gene specific for q-PCR
	qRT-CCD1-Rev	CGGCGGCGACGATTTCAAG	Gene specific for q-PCR
ACTIN	ACTIN-For	CTTGCACCAAGCAGCATGAA	Reference gene in q-PCR
	ACTIN-Rev	CCGATCCAGAACATGTACTTCCTT	Reference gene in q-PCR
UBQ	UBQ10-For	GCTCCGACACCATCGACAACG	Reference gene in q-PCR
	UBQ10-Rev	CTGAGGACCAAGTGGAGGGTGGA	Reference gene in q-PCR
PSY	PSY-For	TGCGGTGAAGTTTGCGCTGA	Gene specific for q-PCR
	PSY-Rev	TGAAGCATTTGGCCCATCCA	Gene specific for q-PCR
bLYC	bLYC-For	TGGTAGCGCTGCTCTTTTGGA	Gene specific for q-PCR
	bLYC-Rev	ACCAGCAGGACCACCACCA	Gene specific for q-PCR
PDS	PDS-For	GTCGGTCACGCGCTCAGGTA	Gene specific for q-PCR
	PDS-Rev	CGAGATGCTGACATGGCCAGA	Gene specific for q-PCR
ZDS	ZDS-For	CCATCGTCACGAGGCCTAGAA	Gene specific for q-PCR
	ZDS-Rev	TGTGTATGAACCGGCGAGGA	Gene specific for q-PCR
BCH1	BCH1-For	GGCACGCTTCTCTATGGAATATGCATGA	Gene specific for q-PCR
	BCH1-Rev	GAATCCATAAGAGAGGAGACCAATCGCT	Gene specific for q-PCR
LUT1	LUT1-For	CGAAATCCCAATCATGGGTCA	Gene specific for q-PCR
	LUT1-Rev	GCACCTCCGAGGAGATCAGC	Gene specific for q-PCR
ZEP	ZEP-For	ATGACCGGCTTCGAGAGTGG	Gene specific for q-PCR
	ZEP-Rev	TTCCGACGATGCAAGGTTGA	Gene specific for q-PCR
VDE	VDE-For	ACCGCTCCGCTGTTGCTAAA	Gene specific for q-PCR
	VDE-Rev	TGGCAATGCACTTTGCGAGT	Gene specific for q-PCR
ABA2	ABA2-For	ACGGTTGATGATGTAGCGAACGCTGTT	Gene specific for q-PCR
	ABA2-Rev	CATCTGAAGACTTTAAAGGAGTGGTTAG	Gene specific for q-PCR

transcription reaction was performed using one µg total RNA and qScript^{TM} cDNA SuperMix (Quanta Biosciences, Mississauga, ON). The cDNA was diluted with sterilized distilled water (1:3), a total volume of 10 µl containing 0.2 µM for each forward and reverse primer (Table 2.1), 1X perfecta SYBR Green FastMix (Quanta Biosciences, Mississauga, ON), and 2 µl cDNA was used in each qRT-PCR reaction. For each line, three biological and three technical replicates were used. The PCR was performed in two steps; 95 °C for three minutes followed by 45 cycles at 95 °C for 10 sec and 58 °C for 30 sec using gene specific primers (Table 2.1). Two reference genes Actin2 *(Act2; AT3G18780)* and Polyubiquitin *(UBQ10; AT4G05320)*, were used to normalize the transcript levels. Transcript levels of the respective genes were analyzed using relative quantification by the comparative Ct method (Schmittgen & Livak, 2008).

2.2.5 Carotenoid analysis

Frozen ground tissue from leaves (100 mg) was used for extraction of carotenoids using limited light and controlled temperature to minimize degradation and isomerization of carotenoids according to the method described by (Yu et al., 2012).

Profiles of individual carotenoids were determined in acetonitrile/dimethylchloride/methanol mixture by HPLC, using a HPLC-DAD system (Agilent Technologies 1200 series). Carotenoid separation was conducted using a YMC 38 "Carotenoid Column" - reverse phase C_{30}, 5 µm column (4.6 × 250 mm; Waters Ltd, Mississauga, ON) with a column temperature of 35 °C by a gradient elution of methanol and tert-methyl butyl ether. The elution started with a mix of 95% methanol and 5% tert-methyl butyl ether, followed by a linear gradient to 35% methanol and 65% tert-methyl butyl ether in twenty five min. The flow rate was 1.2 ml/min. Carotenoids were identified based on their retention times and UV spectra as compared to authentic carotenoid standards (lutein and β-carotene) obtained from CaroteNature (Switzerland).

Ground fresh plant leaf sample (0.5 g) was vortexed vigorously with 10 ml of 80% acetone solvent. The mixture was centrifuged at 10,000 rpm for 15 min at 4°C. 0.5 ml of the supernatant was mixed with 4.5 ml of the solvent and was analyzed for total carotenoids using absorption spectroscopy. The total carotenoid content was calculated by the following equations:

Chlorophyll a (μg/ml) $= 12.25\ A_{663.6} - 2.25\ A_{646.6}$

Chlorophyll b (μg/ml) $= 20.31\ A_{646.6} - 4.91\ A_{663.6}$

Total carotenoids (μg/ml) $= \dfrac{1000A_{470} - 2.27\ (\text{Chl a}) - 81.4\ (\text{Chl b})}{227}$

The factor for multiplying the absorbance values is based on the specific extinction co-efficient of Chlorophyll a and Chlorophyll b.

2.2.6 Plant volatile analysis

Volatile organic compounds (VOCs) were collected from five week old plants over a 48 h period by a process referred to as dynamic headspace collection, following a standard procedure (Cáceres, 2015). Fresh plants were confined in glass chambers with two connection ports (one inlet at the bottom and one outlet at the top). Before use, the chambers were purged clean of any residual VOCs with activated charcoal. Compressed air was allowed to flow through the chambers at 100 ml/min. A Porapak Q 75/150 polydivinylbenzene column (Cat. # 226-115; SKC Inc., USA) was connected at the outlet port of the chamber to collect the volatiles. Every collection included transgenic and WT plants. After collection, the samples were immediately eluted from the Porapak Q with 3 ml HPLC grade dichloromethane (DCM). The eluent was then concentrated to approximately 0.25 ml by passing the samples under a stream of nitrogen gas. The internal standard 2-octanone was added to the samples at a final concentration of 20 μg/ml.

The samples were separated using a DB-5MS + DG 30 m + 10 m Duraguard × 0.25 mm i.d.; film thickness 0.25 μm column (Agilent Technologies, Mississauga, ON), with Helium as the carrier gas and flow rate of 1.2315 ml/min. Analysis was performed using an Agilent Technologies 7890A gas chromatograph equipped with an Agilent Technologies 5975C inert XL EI/CI MSD Triple-Axis Detector. The temperature gradient started at 30 °C for 1 min, then increased at 5 °C/min to 200 °C, and held for 1 min. The total run time for each sample was 36 min. Two microliters of plant volatile samples were injected using an auto sampler into the gas chromatograph (GC) in the pulsed splitless mode (25 psi until 0.5 min; the purge flow to the split vent was 40 ml/min for 1 min). Volatile compounds in the samples were identified by comparison of the mass spectra obtained from authentic standards and additionally confirmed with MS data from the NIST11 and W8N08 libraries (John Wiley and Sons, Inc., New York, NY). Analysis of the volatile profiles was performed using the AMDIS_32 software (version 2.68; Jan 28 2010; Build, 126.47). The authentic standards used were: 1) β-ionone 2) oxoisopherone 3) β-ionol 4) α-ionone 5) β-damescenone 6) theaspirane 7) isophorone 8) caryophyllene 9) limonene 10) dihydro-β-ionone and 11) β-cyclocitral (Sigma Aldrich).

2.2.7 Oviposition choice tests

To test whether the differences in carotenoid-derived volatiles in the different genotypes have an effect on oviposition preference, *T. ni* moths were used in a two plant oviposition choice assay. All moths were used only once for each assay and each assay was replicated three times for each transgenic line (three lines for *CCD1* and three for *CCD4*).

Each plexiglass container (35 x 32 x 32 cm) held five week old plants from two different genotypes (one WT and one transgenic), placed equidistant from each other and from the walls

of the container. Plants used in these trials were matched for size, number of leaves and number of flowers. A total of five male and five female two day old *T. ni* moths were released into each container. The moths were allowed to mate and oviposit freely between the two plants for three days at 24 °C, 55% rh, and 16L:8D photoperiod. Moths were provided with a 5% honey water solution in a plastic bottle with a paper wick placed between the two test plants. At the end of 3 days, the moths were removed and the number of eggs oviposited on each plant were counted.

To evaluate whether the moths differentiated between transformed (CCD) and non-transformed (WT) plants, a no choice experiment using the same protocol was conducted where moths were presented with two plants of the same genotype. Furthermore, the spatial distribution of eggs on the leaves of the plants was subjectively observed. No numerical data was recorded for these observations.

2.2.8 Feeding choice tests

The role of plant carotenoid-derived volatiles in resistance or attraction to feeding by chewing insects can be evaluated in choice or no choice tests. The *T. ni* larval choice and no choice bioassays were conducted with full plants at the four week old vegetative stage, to allow for maximum foliage. A pot with two WT and two transgenic plants (positioned horizontally in the pot) was presented to 16 sec instar *T. ni* larvae, which were starved for 2 h. The bioassay was conducted for a period of 24 h at 24 °C, 55% rh, and 16L:8D photoperiod. At the end of the 24 h period, the larvae were removed and the leaf consumption was estimated. The % leaf damage (x) was scored as follows: (slightly modified from (Hallett et al., 2005)): 0% (score=0), ≤5% (1), 5<x≤20% (2), 20<x≤50% (3), 50<x<100% (4), 100% (5). A similar no choice experiment was

performed with four plants of the same genotype in a pot, to evaluate whether the larvae differentiated between transformed and non-transformed plants.

2.2.9 Statistical analysis

For each genotype, at least three independent lines and three biological replicates (3 plants) per individual line were used for morphological characterization. Molecular characterization involved the use of three technical replicates. One-way analysis of variance (ANOVA) was used to test the difference between the means.

To test the main prediction that female oviposition is influenced by the carotenoid-derived volatiles, a one-way ANOVA was performed on oviposition preference index (OPI) data. This index was calculated as (X-Z)/(X+Z), where X and Z represent the number of eggs laid by the females on each of the two genotypes (X and Z / WT and transgenic) used in the two choice oviposition assay. The oviposition index is useful because it allows the transformation of a categorical variable to a quantitative variable, that can be analyzed using an ANOVA approach (Ryan & Bidart-Bouzat, 2014). The OPI values range from -1 to +1, with values closer to 1 indicating most eggs were laid on X, and those closer to -1 indicating most eggs were laid on Z.

2.3 Results

2.3.1 Molecular characterization of transgenic Arabidopsis plants

A number of independent *CCD* transgenic plants were produced by *Agrobacterium*-mediated transformation. Ten day-old putative transformants were identified on Murashige and Skoog Basal salt mixture (MS) (Phyto Technology Laboratories, U.S.A) based on their resistance to the antibiotic hygromycin B. PCR amplification of the transgene using one vector-specific primer

(CaMV 35S promoter-specific primer) and one transgene specific primer (CCD1-Rev and CCD4-Rev, respectively) further confirmed the presence of the transgene in the transformants (Figure 2.1). A comparative study of gene expression patterns in the transgenic and WT plants was done using qRT-PCR to identify plants with higher levels of transgene expression. Three transgenic lines for each genotype (CCD1 and CCD4) were used for the gene expression study. Transgenic CCD1 plants showed approximately a 20-fold increase in the *CCD1* transcript level in comparison to WT plants ($df=3,8$; $F=4.54$; $P<0.0002$) whereas transgenic CCD4 plants showed approximately a 3.5-fold increase in the corresponding transcript levels ($df=3,8$; $F=7.92$; $P<0.0001$) (Figure 2.2A and 2.2B). No differences in plant morphology were observed in any of the transgenic plants compared to WT at the seedling stage (Figure 2.3A) and later developmental stages (Figure 2.3B).

2.3.2 Effect of *CCD* overexpression on leaf carotenoid levels

The individual carotenoid levels in the transgenic plants showed variable levels over different transgenic lines (Figure 2.4A-E). Noticeably, a significant decrease in lutein content was observed in the CCD1 transgenic plants ($df=3,8$; $F= 11.48$; $P<0.003$) (Figure 2.4A). However, the total carotenoid content in the leaves of the transgenic overexpressing CCD1 and CCD4 plants (measured by absorption spectroscopy) exhibited no significant change in comparison to the untransformed WT control plants (Figure 2.4C).

2.3.3 Effect of *CCD* overexpression on major carotenoid biosynthesis genes

Alteration of expression of some carotenoid biosynthetic genes has been shown to affect the transcript levels of other endogenous carotenoid genes in plants (Diretto et al., 2006). Given the

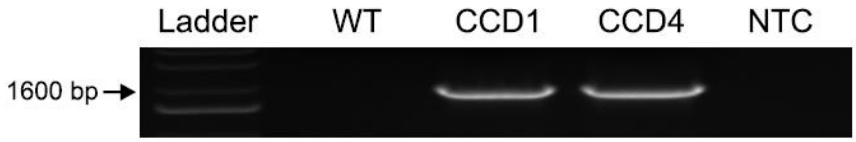


Figure 2.1 Genotyping of CCD1 and CCD4 transgenic Arabidopsis plants. PCR products showing *CCD1* and *CCD4* transgenes in individual transgenic plants. The WT and NTC (no template control) serve as negative controls.

variability of the carotenoid content in Arabidospis leaves, we decided to examine the expression levels of key carotenogenic genes. The expression of nine biosynthetic genes in leaves of the transgenic plants compared with the non-transformed WT control plants is shown in Figure 2.5. Transcript levels of *PSY, PDS, LUT1, VDE* and *ZEP* were higher in the CCD plants (both CCD1 and CCD4) relative to WT ($df=3,8$; $F= 11.48$; $P<0.003$). Transcript levels of *BCH1*, *ABA2* and *ZDS* showed no significant differences between WT and CCD plants, while *bLYC* showed a decrease in CCD plants.

2.3.4 Enhanced volatile emissions from CCD transgenic plants

In total, 22 VOCs from WT plants were identified by matching the mass spectra of each component with the database. These include: aromatic compounds, unsaturated hydrocarbons, monoterpene, sesquiterpenes and the apocarotenoid compound β-ionone. The CCD1 and CCD4 transgenic plants showed a similar profile to the WT plants with respect to their volatile compound profiles (Figure 2.6). After further analysis of the data, a number of semi-quantitative differences were observed between the three genotypes (Table 2.2). The majority of the VOCs were different between the transgenic and the WT controls, but most of these VOCs were aromatic compounds. For the purpose of this research, focus was more on the apocarotenoids and a few monoterpenes and sesquiterpenes. Caryophyllene, a sesquiterpene was 2-fold higher in CCD1 transgenic lines when compared to WT and CCD4 plants and β-ionone, an apocarotenoid showed a 2-fold increase in CCD1 overexpression lines. Sesquiterpenes β-chamingrene and isocaryophyllene showed a 3.5-fold increase in CCD1 and CCD4 transgenic plants and the sesquiterpene humulene levels significantly increased in both sets of transgenic lines.

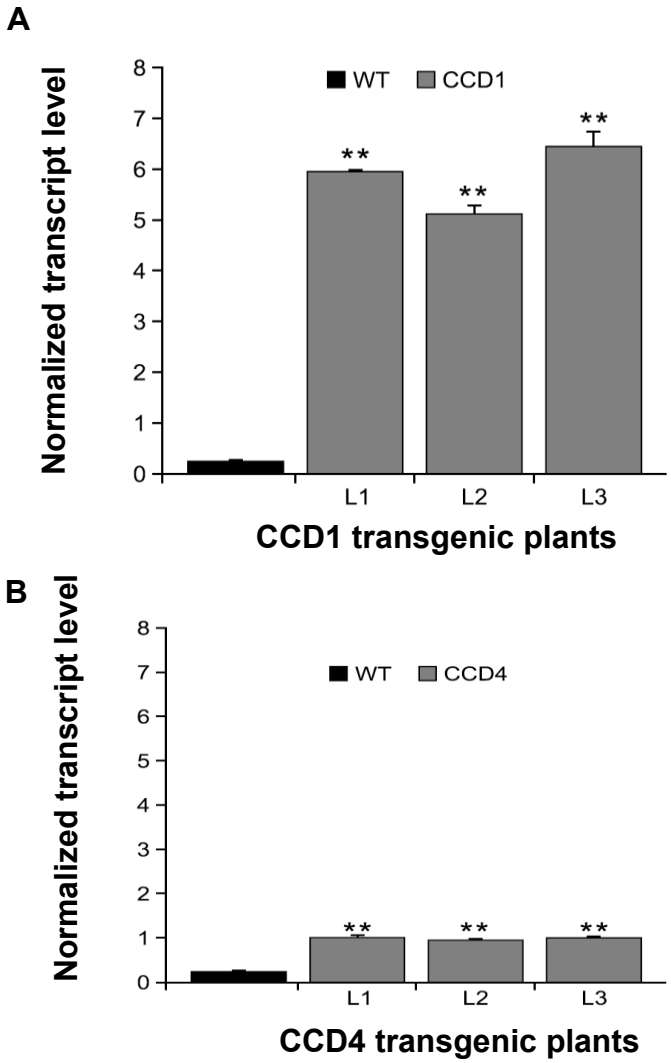


Figure 2.2 Expression profiles of *CCD1* and *CCD4* in Arabidopsis leaves. (A) Level of *CCD1* transcripts in leaves of four week old transgenic seedlings. (B) Level of *CCD4* transcripts in leaves of four week old transgenic seedlings. Asterisks indicate average ± SE (n=3) are significantly different from non-transformed WT control plants at $P<0.05$ (*) or $P<0.01$ (**) using one way ANOVA test. L1, L2 and L3 represent individual transgenic lines.

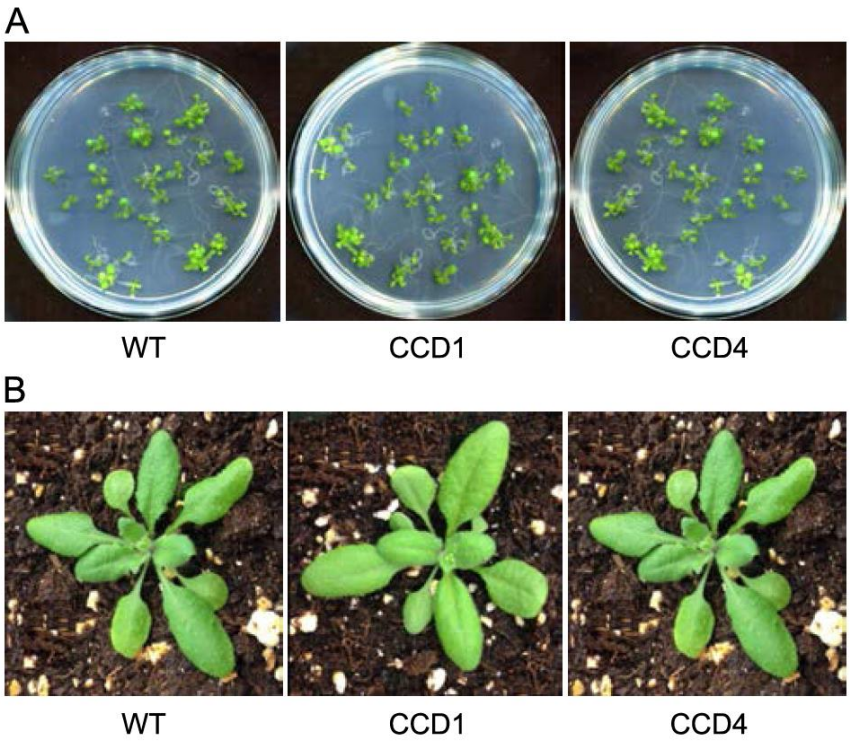


Figure 2.3 Phenotypic comparison of CCD transgenic Arabidopsis plants and WT. (A) Two week old untransformed WT and transgenic seedlings; (B) Four week old untransformed WT and transgenic seedlings

Corrigendum

Date: 23 November 2016

Corrections of substantive errors in master's thesis

The author and primary supervisor have noted errors with Figures 2.3 and 2.6 in this thesis.

Figure 2.3 contains the same photo repeated in error several times. This experiment has been repeated to confirm that transformation of WT Arabidopsis plants with *CCD1* and *CCD4* genes does not result in a changed phenotype. The new data is presented in the figure below.

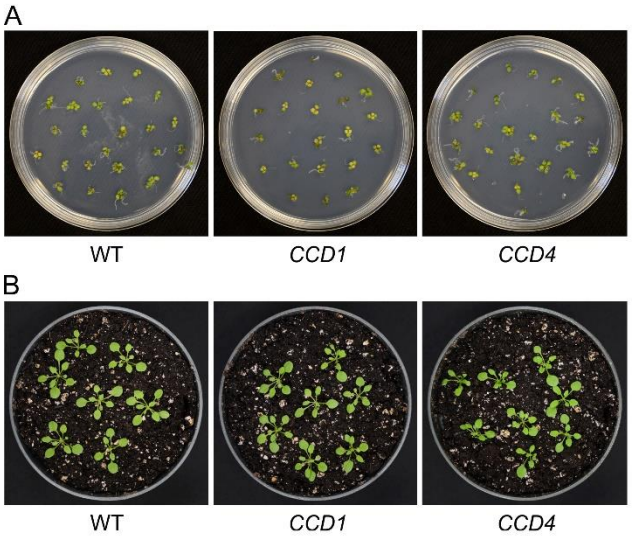


Figure 2.3 Phenotypic comparison of CCD transgenic Arabidopsis plants and WT. (A) Two week old untransformed WT and transgenic seedlings on agar plates containing Murashige and Skoog salts; (B) Four week old untransformed WT and transgenic seedlings on plates containing ProMix BX potting soil.

The example chromatograms in Fig. 2.6 come from the same plants, and are the same data as those presented in Fig. 3 of Cáceres et al 2016 Repellent and attractive effects of α-, β- and dihydro-β- ionone to generalist and specialist herbivores. J Chem Ecol, 42:107-117, and should have been attributed appropriately.

These errors do not change the conclusions of the thesis, nor were they a result of deliberate data manipulation. We apologize for any inconvenience caused.

Author: Sneha Challa

Primary Supervisor: Dr. Abdelali Hannoufa

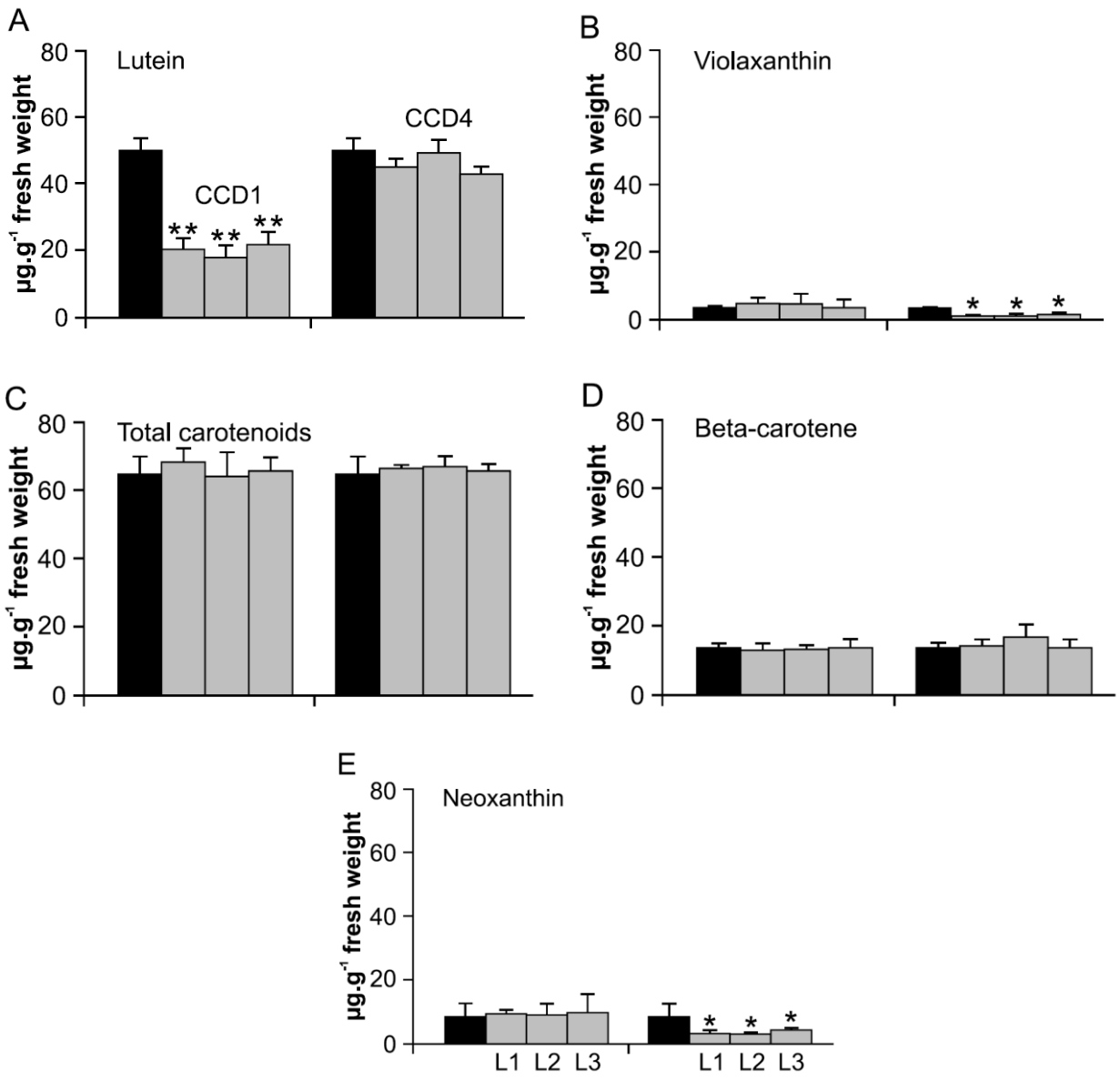


Figure 2.4 Effect of overexpression of *CCD1* and *CCD4* on leaf carotenoids in Arabidopsis. Levels of (A) Lutein, (B) Violaxanthin, (C) Total carotenoids, (D) β-carotene, (E) Neoxanthin. Black bars represent WT plants and the grey bars represent the different CCD transgenic lines. Asterisks indicate average ± SE (n=3) are significantly different from WT control plants at $P<0.05$ (*) or $P<0.01$ (**) using one way ANOVA test.

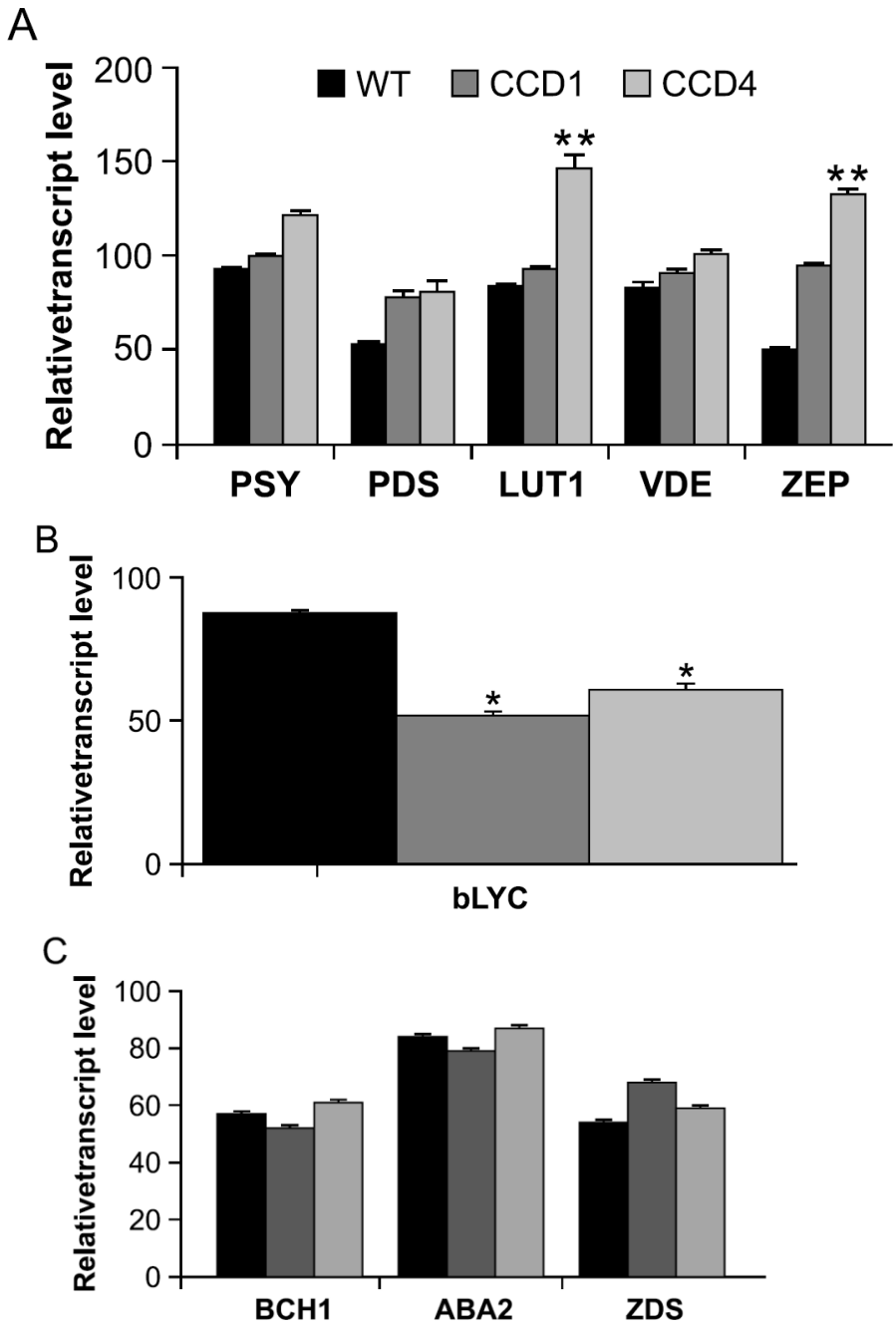


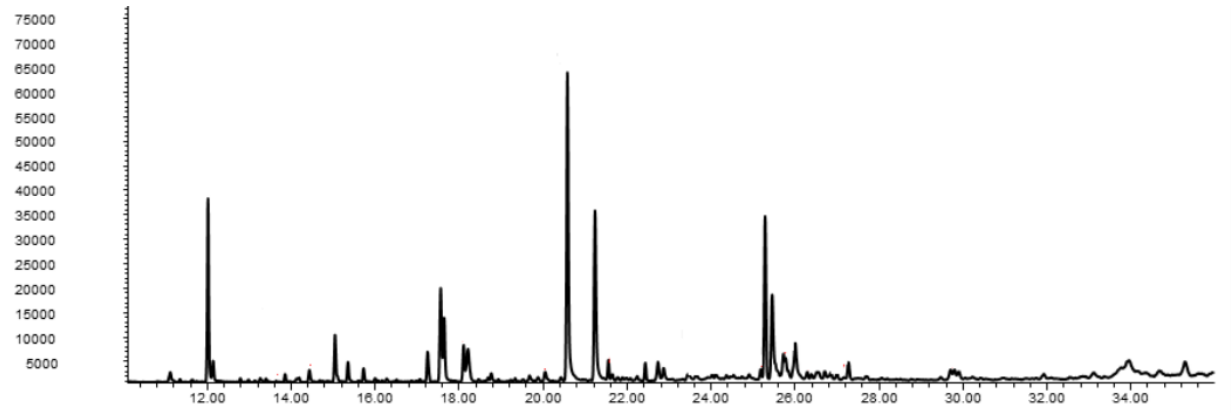
Figure 2.5 Effect of *CCD4* and *CCD1* over-expression on transcript levels of some carotenogenic genes in Arabidopsis leaves. Asterisks indicate average ± SE (n=3) are significantly different from non-transformed WT control plants at P<0.05 (*) or P<0.01 (**) using one way ANOVA test. Black bars represent WT plants, dark grey bars represent CCD1 and the light grey bars represent CCD4 plants.

Table 2.2 Volatile profiles of WT and CCD Arabidopsis plants

		WT			*CCD1*			*CCD4*		
	Compounds	**Average**	**±**	**S.D.**	**Average**	**±**	**S.D.**	**Average**	**±**	**S.D.**
1	Benzaldehide	0.64	±	0.03	0.22**	±	0.04	0.02**	±	0.01
2	2,4-Nonadiyene	0.57	±	0.01	0.17**	±	0.1	nd	-	-
3	3-undecyne	1.07	±	0.04	0.01**	±	0.01	0.12**	±	0.02
4	Benzene,1,4-diethyl	0.25	±	0.03	0.22	±	0.06	0.12**	±	0.01
5	Acetophenone	1.23	±	0.03	0.39**	±	0.03	0.45**	±	0.04
6	Benzene,1-methyl-3-ethyl	1.78	±	0.28	1.62*	±	0.25	1.38**	±	0.11
7	Benzaldehide,4-ethyl	4.91	±	0.32	3.44**	±	0.33	5.31**	±	0.36
8	Isoxylaldehyde	2.05	±	0.08	1.31**	±	0.16	1.8*	±	0.16
9	3-cyclohexene-1-ol-5-methylene-6-(methylethenyl)	0.75	±	0.05	0.67	±	0.04	0.67	±	0.07
10	Acetophenone,2,4-dimethyl	15.43	±	0.61	13.03*	±	0.78	15.69	±	1.06
11	Acetophenone-4-ethyl	8.56	±	0.71	6.84*	±	0.46	9.44	±	0.69
12	3-buten-2-one,4 phenyl	0.29	±	0.03	0.17*	±	0.04	0.24	±	0.06
13	Ethanone-1-(2,3-dihydro-1H-inden-5-yl)	0.43	±	0.04	0.35*	±	0.04	0.51	±	0.07
14	alpha-cubebene	1.66	±	0.02	0.43**	±	0.02	0.37**	±	0.06
15	alpha-thujene	0.85	±	0.06	1.91**	±	0.2	2.28**	±	0.25
16	Caryophyllene	6.01	±	0.52	12.83**	±	0.39	7.19*	±	0.77
17	Thujopsene	2.48	±	0.06	4.21**	±	0.95	1.18	±	0.07
18	Humulene	0.04	±	0.01	0.71**	±	0.08	0.2**	±	0.04
19	beta-ionone	0.34	±	0.05	0.57**	±	0.06	0.42	±	0.02
20	beta-chamingrene	0.3	±	0.04	1.11**	±	0.2	1.16**	±	0.19
21	Isocaryophyllene	0.21	±	0.04	0.78**	±	0.05	0.52**	±	0.04
22	Caryophyllene epoxide	0.41	±	0.03	0.55*	±	0.07	0.4	±	0.04

Average ± S.D. represents the relative peak area for each compound and is an average of at least three biological replicates. Relative peak area was calculated as a ratio of peak area of each compound to the peak area of the internal standard, 2-octanone. Asterisks indicate average ± S.D. are significantly different from WT control plants at $P<0.05$(*) or $P<0.01$(**) using one way ANOVA. nd indicates values that were not detected.

A)



B)

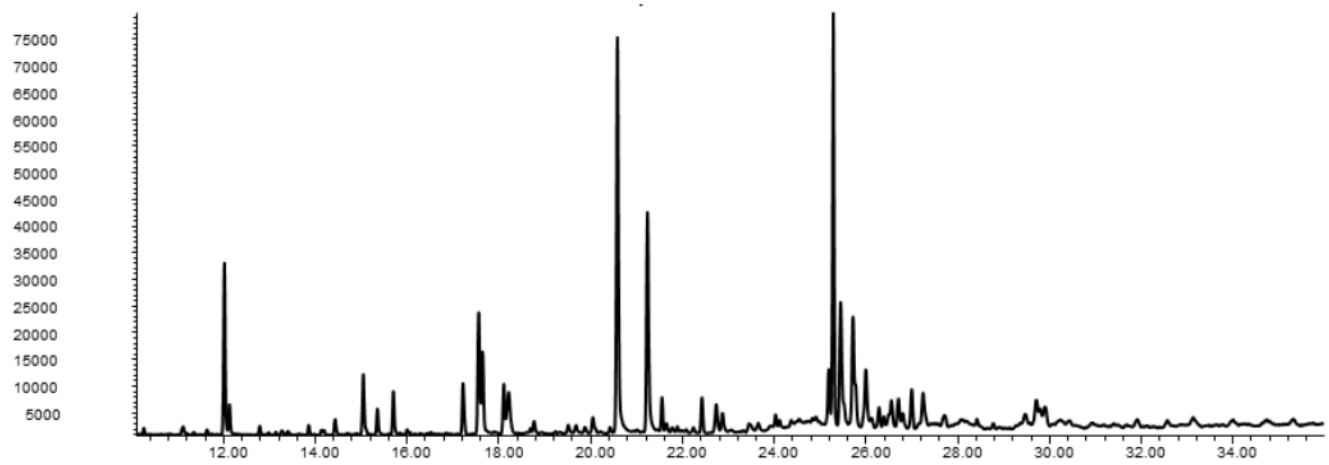


Figure 2.6 Headspace analysis of volatiles collected from flowering Arabidopsis plants. A) Representative GC-MS total ion chromatograms showing volatile compounds exuded by WT control plants and B) Representative GC-MS total ion chromatograms showing enhancement of certain volatile compounds in CCD plants

Table 2.3 Total number of eggs oviposited by *T. ni* females on WT and transgenic Arabidopsis plants in a choice assay

		Eggs laid							
Gene	**Genotype**	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**Average ± S.D.**	**OPI**
***CCD*1**	WT	215	164	219	254	132	321	218 ± 67	-0.35
	L1	396	365	456	541	401	612	462 ± 96	
	WT	252	241	101	415	131	213	226 ± 111	-0.29
	L2	512	524	321	390	325	421	416 ± 88	
	WT	101	342	345	298	301	364	292 ± 97	-0.34
	L3	312	685	564	525	782	701	595 ± 167	
***CCD*4**	WT	123	201	152	295	251	177	200 ± 64	-0.38
	L1	422	522	428	529	408	392	450 ± 60	
	WT	396	246	191	326	285	215	277 ± 76	-0.26
	L2	528	396	350	703	503	373	476 ± 133	
	WT	164	149	279	224	230	215	210 ± 47	-0.34
	L3	316	367	557	556	452	345	432 ± 106	

R1, R2, R3, R4, R5 and R6 represent the six replicates used in the study. L1, L2 and L3 represent the different transgenic lines for each genotype. Values are the number of eggs laid on each plant. OPI is oviposition preference index. Negative values and values closer to zero, indicate a preference towards transgenic plants.

2.3.5 Oviposition preference for transgenic plants with enhanced volatile emissions

The differences in constitutive volatile emissions between different Arabidopsis lines had different effects on oviposition preference (Table 2.3). A positive oviposition preference (OPI) for CCD plants over WT was observed. These results were further corroborated by little or no significant difference in the OPIs from the no choice assays (Table 2.4). The female moths did not discriminate between two plants of the same genotype. From the subjective observations made for the spatial distribution of eggs, the eggs on the transgenic CCD plants were found in tight clusters of 10 or more whereas the WT plants had the eggs dispersed over the entire leaf surface. The eggs on the WT plants were usually singly deposited or were in loose clusters of less than 5.

2.3.6 Feeding damage by *T. ni* larvae on transgenic CCD plants

The results of closed-chamber bioassay experiments using both CCD1 and CCD4 transgenic plants consistently showed no difference in the leaf area consumed by the larvae (Table 2.5). Leaf damage was not limited to rosette leaves alone as it was found in cauline leaves as well. This whole plant damage indicates that the larvae do not exhibit feeding preference for a specific genotype, as opposed to statistically significant oviposition preference for both the CCD1 and CCD4 transgenic genotypes over WT.

2.4 Discussion

This study provided experimental evidence that altered volatile profiles caused by overexpression of *CCD* genes can influence female oviposition behavior in cabbage looper moths. Others have reported that defense-related secondary chemicals produced by different

plant species, such as glucosinolates and their hydrolysis products, influence oviposition of insect herbivores (Badenes-Perez et al., 2014; Gols, 2014; Halkier & Gershenzon, 2006; Ryan & Bidart-Bouzat, 2014) and specifically how variation in the secondary chemistry of Arabidopsis influences oviposition (De Vos et al., 2008; Sun et al., 2010). However, to our knowledge, the effect of carotenoid-derived volatiles on oviposition behavior of *T. ni* moths has not yet been investigated. In this study, it was demonstrated that *T. ni* exploits carotenoid-derived plant volatiles to locate favorable hosts for oviposition. *T. ni* moths oviposited significantly more eggs on transgenic *A. thaliana* plants that produced higher amounts of the apocarotenoid β-ionone and the sesquiterpene caryophyllene, compared to WT controls. These results suggest that female oviposition preferences were guided by volatile cues from the plants. Conversely, a no choice experiment with plants of similar genotype (two WT or two transgenic plants), did not show a significant difference in the number of eggs on each plant. This finding supports our hypothesis that moths use volatile cues to guide their oviposition choice.

The potential attractiveness of *T. ni* to transgenic plants could be explained by an intuitive expectation that adults lay their eggs where offspring performance is optimal and this expectation has been termed the 'preference-performance' hypothesis or 'mother-knows-best' hypothesis (Clark et al., 2011; Jaenike, 1978; Valladares & Lawton, 1991). According to this hypothesis, the increased egg deposition recorded on transgenic plants can be interpreted as the recognition of these plants as favorable by the *T. ni* moths thereby implying the possible attraction towards higher β-ionone and caryophyllene levels being produced by the transgenic plants. Conversely, another interpretation of why the moths oviposit more on the transgenic plants could be to increase the chances of offspring survival on the putatively unfavorable transgenic plants. We cannot rule out the possibility that the moths detect the transgenic plant

Table 2.4 Total number of eggs oviposited by *T. ni* females on WT and transgenic Arabidopsis plants in a no choice assay

		Eggs laid							
Gene	**Genotype**	**R1**	**R2**	**R3**	**R4**	**R5**	**R6**	**Average ± S.D.**	**OPI**
	WT	79	164	124	158	67	108	117 ± 40	-0.02
	WT	68	154	156	191	56	104	122 ± 54	
CCD1	L1	145	116	130	156	146	111	135 ± 18	0.07
	L1	164	115	178	110	167	191	154 ± 34	
	L2	179	65	151	165	116	124	133 ± 41	0.0
	L2	157	54	172	115	145	156	133 ± 43	
CCD4	L3	164	163	167	130	164	146	156 ± 15	0.04
	L3	124	156	125	145	145	162	143 ± 16	
	L1	264	191	130	114	125	106	155 ± 61	-0.004
	L1	217	157	110	201	200	119	167 ± 46	
	L2	315	82	76	164	149	130	153 ± 87	0.01
	L2	350	84	65	156	157	124	156 ± 102	

R1, R2, R3, R4, R5 and R6 represent the six replicates used in the study. L1, L2 and L3 represent the different transgenic lines for each genotype. Values are the number of eggs laid on each plant. OPI is oviposition preference index. Negative values and values closer to zero indicate a preference towards the transgenic plants.

Table 2.5 Larval feeding damage in vegetative WT and transgenic Arabidopsis over-expressing *CCD* genes.

		Average ± S.D.	
Gene	**Line**	**Wild-type plant score**	**Transgenic plant score**
	L1	4 ± 0	4 ± 0
CCD1	L2	4 ± 0	3 ± 0.57
	L3	3 ± 0	3 ± 0
	L1	3 ± 0	4 ± 0.57
CCD4	L2	3 ± 0	2 ± 0.57
	L3	3 ± 0	3 ± 0

The values represent the average score (for three replicates each) attributed to the extent of leaf feeding damage.

be unfavorable and hence deposit more eggs to match the larval survival rate on transgenic plants to the survival rate on WT control. This explanation is supported by the differences in the spatial distribution of the eggs on the plants of different genotypes, found in our study. Transgenic plants showed the presence of tight clutches of at least 10 to 15 eggs whereas WT plants mostly showed single eggs dispersed throughout the plant. This difference in the pattern of egg distribution could be indicative of the potential unfavorability of the enhanced carotenoid-derived volatiles from the transgenic plants.

Assuming that adult performance is indicative of offspring performance, investigating larval feeding on both the transgenic and the WT plants was completed to better understand the female oviposition preferences. The larvae fed on both WT and transgenic plants to the same degree, thereby indicating that larvae are not sensitive to olfactory cues or affected by any nutritional differences between genotypes.

Some studies have suggested that generalist insects are not attracted to, or even repelled by, plant secondary metabolites (Wittstock et al., 2003). However, our results show that the generalist *T. ni* appears to distinguish between CCD and WT plants. Furthermore, even though oviposition experiments with CCD1 and CCD4 overexpression plants emitted enhanced levels of β-ionone and caryophyllene, we cannot completely rule out the possibility that other individual volatiles or blends of volatiles from the plants also contribute to the oviposition choice. Further work using individual volatiles may provide further evidence for the role of specific volatile compounds in insect host location for oviposition and feeding.

Manipulating the transcript levels of the *CCD* gene accounts for a majority of the variation observed in the carotenoid content among individual plants (Auldridge et al., 2006a; Auldridge et al., 2006b; Harrison & Bugg, 2014; Messias et al., 2014). But, these genes are not the only

factors responsible for determining carotenoid levels. Studies have shown that both light and nutrient availability play a role in determining the carotenoid content in plants at any given point of time (Fanciullino et al., 2014; Logan et al., 1996; Soran et al., 2014; Thayer & Björkman, 1990). Contrary to the common assumption that light and nutrient availability are two independent factors regulating the carotenoid content in plants, an interaction between light and nutrient availability was found (Valladares et al., 2000). The total carotenoid content was higher in nutrient-limited than in nutrient-rich plants grown in the sun, whereas the reverse was true for their shade counterparts. Despite our best efforts to control these factors and keep them constant for the different plants used throughout the study, it is possible that discrepancies have crept in. Hence, the putative variation in the carotenoid content may be a result of the action of the *CCD* genes combined with some unknown abiotic factors.

Results reported here may have implications not only for the evolutionary ecology of chemically mediated plant-insect interactions but also for pest management, as Arabidopsis is a model plant belonging to the economically important Brassicaceae family and shares the same chemical defense system with many crucifer crop species (Bidart-Bouzat & Kliebenstein, 2008; Björkman et al., 2011). Different volatiles may influence oviposition rates of different insects in many ways, which in turn may affect plant damage levels in both natural and agricultural systems. In our study, moths appeared to discriminate between different levels of volatiles thereby providing evidence for the olfactory sensitivity of moths, though more experiments with individual synthetic chemicals are necessary to interpret the results decisively. Information on the potential effects of different volatiles in varying concentrations is therefore important for selecting a more effective pest management strategy, particularly against devastating crucifer pests, such as *T. ni*.

Given the possibility that the seemingly positive attraction of the moths to the transgenic plants might be a safety mechanism to increase the chances of larval survival as well as a preference for the compound, further confirmation is necessary by investigating the larval performance. Although, the young larvae do not show any preference for a particular genotype it will be of great interest to study the fitness consequences of the newly hatched larva, in order to gain a more complete understanding of the role of the carotenoid-derived volatiles in larval development and adult moth reproduction. Nevertheless, by demonstrating that enhanced β-ionone and caryophyllene emission by *CCD1* and *CCD4* overexpression in Arabidopsis attracts *T. ni* moth oviposition, I have strong evidence for the influence of carotenoid-derived volatiles on oviposition behavior of pests. Pending field tests, these transgenic plants producing higher levels of specific volatiles can be used as a trap crop to attract the pests away from the main agricultural produce. This strategy would be ideal as it would cater to growing consumer demands for non-transgenic and chemical free food crops.

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Chapter 3: Effect of carotenoid-derived volatiles on insect oviposition and feeding preferences in tomato

3.1 Introduction

Carotenoids are a family of over 600 different plant pigments synthesized by all photosynthetic organisms as well as by some fungi and heterotrophic bacteria (Ilg et al., 2010). Carotenoids are multifaceted compounds with a wide range of functions in plants as well as humans. In plants, the myriad roles of carotenoids include photoprotectants, antioxidants and accessory pigments in photosynthesis. Carotenoids also serve as substrates for the synthesis of apocarotenoids, biologically active derivatives formed by oxidative cleavage (Gonzalez-Jorge et al., 2013). Apocarotenoids include vitamin A, the plant hormones abscisic acid and strigolactones, and a wide range of volatile compounds that serve as attractants for pollinators and herbivores (Heil, 2014; Heil & Bueno, 2007). In addition, it has been proposed that these compounds act as mediators of indirect defense because of their demonstrated capacity to attract predators and parasitoids of herbivores (Dicke & Van Loon, 2000; Tumlinson et al., 1999). Important in human nutrition, carotenoids act as antioxidants that protect cells from the danger of free radicals that may be produced by the body during metabolism or by environmental factors such as smoke, pollutants and UV radiation. β-carotene is one of the most well known and most studied carotenoids found in carrots, pumpkin, peaches and sweet potatoes. In the presence of carotenoid cleavage dioxygenases, β-carotene is catabolized into two vitamin A molecules important in the growth and repair of body tissues, formation of bones and teeth and development of healthy eye tissues. Given the dietary and ecological importance of carotenoids, and the fact that humans cannot synthesize carotenoids (Sommer & Vyas, 2012) the enhancement of carotenoid content in

fruits, vegetables and seeds (the primary sources of carotenoids for animals) would be nutritionally beneficial. Engineering the carotenoid pathway to alter the levels of carotenoids has been successfully attempted in a number of studies (Gonzalez-Jorge et al., 2013; Harjes et al., 2008; Wei et al., 2011; Yan et al., 2010). However, investigating the roles and effects of apocarotenoids, especially on insect behavior, is still in its infancy.

Apocarotenoids are synthesized through the oxidative cleavage of carotenoids mediated by carotenoid cleaving dioxygenases (CCDs) (Marasco et al., 2006). The CCD's form a family of enzymes that are further subdivided into NCED (9-cis-epoxycarotenoid dioxygenases) and CCD's based on substrate specificity (Auldridge et al., 2006). *CCD1* gene is one member of the carotenoid cleaving family which catalyzes the cleavage of a broad range of carotenoids to produce volatile aroma compounds such as β-ionone, α-ionone, 3-hydroxy-β-ionone, pseudoionone, geranylacetone, and 6-methyl-5-hepten-2-one (Auldridge et al., 2006; Simkin et al., 2004; Vogel et al., 2008). For example, in *Solanum lycopersicum* L. (Solanaceae) *CCD1* generates flavor volatiles such as geranylacetone, pseudoionone, and β-ionone (Simkin et al., 2004). Silencing of the tomato *CCD1* (*LeCCD1A* and *LeCCD1B*) resulted in a decrease in fruit volatile apocarotenoids, such as β-ionone and geranylacetone, thus suggesting a link between *CCD1* and apocarotenoid production *in vivo* (Simkin et al., 2004). Although there have been functional studies on CCD enzymes expressed in *E. coli* to determine their enzymatic activities and substrate preferences, very few studies have focused on measuring volatiles generated as a result of *CCD* and *NCED* expression. One study showed that transgenic *Arabidopsis thaliana* (Arabidopsis) overexpressing *CCD1* exhibited enhanced levels of β-ionone along with reduced feeding damage by, crucifer flea beetles *Phyllotreta cruciferae* (Coleoptera: Chrysomelidae) (Wei et al., 2011). This suggested that the volatile apocarotenoids deterred the insects from

feeding on these plants. Therefore, considering that manipulating the carotenoid pathway has been shown to affect insect feeding in Arabidopsis (Wei et al., 2011), we set out to investigate the influence of these volatiles on insect behaviour in tomato.

The objectives of the present study were to assess oviposition repellant effects of selected terpene-derived volatiles (Table 3.3) from tomato plants over-expressing *CCD* genes, by measuring changes in: a) the transcript levels of genes involved in volatile terpene synthesis; b) the constitutive and induced volatile emission levels; c) the carotenoid profile and d) the oviposition preference of cabbage looper *Trichoplusia ni* Hubner (Lepidopetera: Noctuidae) and greenhouse whitefly *Trialeurodes vaporariorum* (Hemiptera: Aleyrodidae).

The role of carotenoid cleavage dioxygenases in the regulation of volatile emission was examined by comparing the volatile levels and the insect oviposition preference for untransformed tomato plants with that of LeCCD1-1 and LeCCD1-2 transgenics (which overexpress either carotenogenic gene *LeCCD1-1* or *LeCCD1-2*).

3.2 Materials and methods

3.2.1 Tomato cultivars

Tomato (*Solanum lycopersicum* var Micro-Tom cultivar) is an excellent model plant for genomic research of solanaceous plants some of which are agriculturally important crops including eggplant, potato, pepper and tobacco. To evaluate the effects of altered carotenoid pathway genes on carotenoid metabolism, volatile production and insect oviposition preferences, Micro-Tom cv. tomatoes (WT) and two genetically modified (GM) tomato lines over-expressing the carotenogenic genes *LeCCD1-1* (AY576001) and *LeCCD1-2* (AY576002), the transgenic CCD1-1 and CCD1-2 were designed. The WT tomato cultivars were obtained from Dr.

Vojislava Grbic, University of Western Ontario, London, ON, and have the same genetic background as the transgenic plants. All tomato plants were grown from seed on 0.8% (w/v) agar plates containing Murashige and Skoog salts (Phyto Technology Laboratories, USA). Ten day old seedlings were transferred to pots with ProMix BX potting soil (premier Horticulture, Quackertown, PA, USA) in growth chambers. Plants used for insect bioassays were seven-eight week old.

3.2.2 Cloning of *LeCCD1* and transformation of tomato

The over-expression transgenic genotypes LeCCD1-1 and LeCCD1-2 (Simkin et al., 2004) were generated using 35S::CCD transgene cassettes. The cassettes were constructed by using full length cDNA of *LeCCD1-1* and *LeCCD1-2* genes that were amplified by PCR using primers CCD1-1-For and CCD1-1-Rev (Table 3.1). The fragments were cloned into the Gateway pENTRD vector (the entry vector; Life Technologies) and the resulting constructs were transferred to *Agrobacterium tumefaciens* cells (GV3101 strain; containing rifampicin and gentamycin resistance) using electroporation. The Agrobacterium strain was then used to transform tomato according to the method described by Cruz-Mendívil et al., 2011. Tomato cotyledons were excised from 10 day-old seedlings and cultured on preculture medium followed by infection and co-cultivation medium. Following shoot induction, shoot elongation and root induction, rooted plantlets were transferred to soil and grown in growth chamber under controlled conditions. Transgenic plants were screened for the presence of the transgene by PCR using the forward primer 35SF3-For and the gene-specific reverse primers (CCD1-1Rev or CCD1-2Rev) (Table 3.1).

Table 3.1 List of primer sequences used for PCR and qRT-PCR analysis in this study

Gene	Primer name	Sequence (5′- 3′)	Use of primers
	35SF3	CAATCCCACTATCCTTCGCAAGACCC	35S forward primer for pMDC32
LeCCD1-1	*CCD*1-1-Rev	TCACAGTTTGGCTTGTTCTTGAATTTG	genotyping primer
	qRT-CCD1-1-For	ATGGGAAGCTGTTGGCATT	Real time primer for *LeCCD1*-1
	qRT-CCD1-1-Rev	GTGGGGTGTGAGCATATCCA	Real time primer for *LeCCD1*-1
LeCCD1-2	*CCD*1-2-Rev	TCACATTTTGGCTTGCTCCTG	genotyping primer
	qRT-CCD1-2-For	TAAAGGGCTGTTCGGGTTGT	Real time primer for *LeCCD1*-2
	qRT-CCD1-2-Rev	TTGCAGATCTCCATCCTCCA	Real time primer for *LeCCD1*-2
ACTIN	ACTIN-For	CATGCCATTCTTCGTTTGGA	Real time primer for tomato *ACTIN*
	ACTIN-Rev	GAGCTGCTCCTGGCAGTTTC	Real time primer for tomato *ACTIN*

3.2.3 Insect stocks

Laboratory population of *T. ni* used in this experiment originated from insects maintained by the Forest Pest Management Centre, Natural Resources Canada, Sault St. Marie, ON. Upon transferring to the Southern Crop Protection and Food Research Centre (SCPFRC), Agriculture and Agri-Food Canada (AAFC), London, ON, *T. ni* were reared at 28 °C under a L16:D8 photoperiod on a meridic diet (Chippendale, 1965). The larvae were kept in diet cups in environmental chambers with relative humidity (rh) maintained at 60% (day) and 70% (night). Newly enclosed adults were sorted by sex and kept in separate plexiglass cages in a growth chamber set at 24 ± 1 °C and 55 ± 5% rh, and L16:D8 photoperiod. Adult mature, mixed sex greenhouse whiteflies (*T. vaporariorum*) were obtained from the Greenhouse and Processing Research Centre, AAFC Harrow, ON, and were maintained at 24 ± 1 °C and 60 ± 5% rh, L16:D8 photoperiod. All insects were kept at 4 °C for 30 min prior to being used in assays, to restrict movement and allow ease of handling.

3.2.4 Extraction and determination of carotenoids

Extraction and measurement of carotenoids by HPLC were performed according to the method described by (Yu, 2012). Briefly, fresh ground leaf and fruit tissue (about 0.2 g) along with 3 ml of ethanol containing 0.1% ascorbic acid (w/v), was vortexed for 20 s, and placed in a water bath at 85 °C for 5 min. The carotenoid extract was saponified with potassium hydroxide (120 µl, 80% w/v) in the 85 °C water bath for 10 min. After saponification, the samples were immediately placed on ice, and cold deionized water (1.5 ml) was added. Carotenoids were extracted twice with hexane (1.5 ml) and centrifuged to separate the layers. Aliquots of the extracts were dried under a stream of nitrogen and dissolved in a 1:1 (v/v) mixture of

dichloromethane/methanol before HPLC analysis. The carotenoids were separated using a YMC 38 "Carotenoid Column" – a reverse phase C30, 5 μm column (4.6 × 250 mm; Waters Ltd, Mississauga, Canada) with a column temperature of 35 °C and a gradient elution of methanol and tert-methyl butyl ether. The elution started with a mix of 95% methanol and 5% tert-methyl butyl ether, followed by a linear gradient to 35% methanol and 65% tert-methyl butyl ether in 25 min. The flow rate was 1.2 ml/min. Carotenoid pigments were identified by comparing the retention time and absorption spectra of individual peaks with standards of lutein and β-carotene (CaroteNature Switzerland). The total carotenoid content of tomato leaves was measured by absorption spectroscopy at 461 and 664 nm (Wellburn, 1994). The carotenoid content (μg/ml) was calculated using the extinction co-eficient equation: $[A_{461} - (0.046 \times A_{664})] \times 4$, and converted to μg/g leaf tissue.

3.2.5 Plant volatile profile

Volatile organic compounds (VOCs) were collected from five week old plants over a 48 h period by air entrainment (also referred to as dynamic headspace collection) following a standard procedure (Cáceres, 2015). Fresh plants were confined in glass chambers with two connection ports (one inlet at the bottom and one outlet at the top). Before use, the chambers were purged clean with activated charcoal. Compressed air was allowed to flow through the chambers at a flow rate of 100 ml/min. A Porapak Q 75/150 polydivinylbenzene column (Cat. # 226-115; SKC Inc., USA) was connected at the outlet port of the chamber to collect the volatiles. Every collection was performed along with a WT control, and after collection, the samples were immediately eluted from the Porapak Q with 3 ml HPLC grade DCM (dichloromethane). The

eluent was then concentrated to exactly 0.25 ml by passing the samples under a stream of nitrogen gas.

The samples were analyzed using an Agilent Technologies Inc. fused silica capillary column (DB-5MS + DG; 5% (w/v) phenylmethyl silicone; 30 m length + 10 m Duraguard × 0.25 mm i.d.; film thickness 0.25 μm) and an Agilent Technologies 7890A chromatograph equipped with an Agilent Technologies 5975C inert XL EI/CI MSD Triple-Axis Detector. The carrier gas used was Helium (12.445 psi; 1.2315 ml/min). The voltage used in the EMV mode was relative and the resulting EMV was 1376. The oven temperature was maintained at 30 °C for 1 min, then increased at 5 °C/min to 200 °C, and then held for 1 min at this temperature. The total run time for each sample was 36 min. Two microliters of plant volatile samples were injected using an auto sampler into the gas chromatograph (GC) in the pulsed splitless mode (25 psi until 0.5 min; the purge flow to the split vent was adjusted at 40 ml/min for 1 min). Volatile compounds in the samples were identified by comparison of the mass spectra obtained from authentic standards and additionally confirmed with mass spectroscopy (MS) data with the NIST08 and W8N08 libraries (John Wiley and Sons, Inc., New York, NY). Analysis of the volatile profiles was performed using the AMDIS_32 software (version 2.68; Jan 28 2010; Build, 126.47). Compounds corresponding to each peak were identified using the National Institute of Standards and Technology (NIST) Mass Spectral database software (version 2.0 f; Build Apr 1 2009).

3.2.6 Dual choice oviposition assay

After collection of HS volatiles for 48 h, the previously described tomato plants were immediately used in an oviposition choice assay to determine whether *T. ni* and *T. vaporariorum* adults discriminate between the transformed and non-transformed plants. One LeCCD and one

WT plant separated by 12 cm were placed on a metal tray with a layer of sand and enclosed by a plastic cover with screen vents in the top. The *T. ni* adults, 5 male and 5 female moths, 2 days post-eclosure were released in the centre of each cage and allowed to oviposit for 3 days. Moths were provided with a 5% honey water solution in a plastic bottle with a paper wick placed between the two test plants. The whitefly oviposition choice assay used 40 *T. vaporariorum* mixed sex adults released into the same sized chamber with one transformed and one non-transformed plant. At least three replicates were tested per tomato line and the height, age and number of flowers per plant was matched. All insects were used only once for each assay. After 3 days, all adults were removed from the chamber, and the number of eggs on each plant and the walls of the chamber were counted. The *T. vaporariorum* adults were sexed only after the experiment, to avoid damage to the insects.

3.2.7 No choice oviposition assay

The objective of this experiment was to determine adult ovipostion preference for 2 plants of the same genotype. All tomato plant genotypes previously listed were tested. Five male and female *T. ni* adult moths were released into each cage containing two potted plants and a plastic bottle of 5% honey water with a wick. Plants were matched for size, age and number of flowers. *T. vaporariorum* assays involved 40 mixed sex adults. After 3 days, adults were removed and the total number of eggs per plant was assessed. The number of replicates per line was at least 4 and the total number of eggs per plant genotype was compared.

3.2.8 Statistical analysis

Statistical differences in the concentration of VOCs released by the WT and transgenic plants and between the transcript levels of the two carotenogenic genes between the transformed and

untransformed WT control plant groups were determined by one-way analysis of variance (ANOVA). Statistical differences for the transformed oviposition deterrence index (ODI) using the following equation, ODI = X-Z/X+Z were determined by two-way ANOVA. All statistical tests were performed using SAS version 9.1 (SAS Institute, Cary, NC, USA).

3.3 Results

3.3.1 Molecular characterization of transgenic tomato plants

Transgenic tomato plants harboring *LeCCD1-1* and *LeCCD1-2* transgenes were generated and analyzed by PCR using primers specific for the 35S promoter and respective *CCD* genes (Table 3.1). Three independent homozygous lines of each of LeCCD1-1 and LeCCD1-2 were obtained and expression of *LeCCD1-1* and *LeCCD1-2* was determined by qRT-PCR (Figure 3.1). Transgenic LeCCD-1 plants showed approximately a 150-fold increase in the Le*CCD1* transcript level compared to WT plants whereas transgenic LeCCD1-2 plants showed approximately a 100-fold increase in the corresponding transcript levels (Figure 3.1A and 3.1B). No differences in plant morphology were observed in any of the transgenic plants compared to WT at 10 week old stage (Figure 3.2).

3.3.2 Effect of *CCD1* overexpression on leaf carotenoid content

Comparison of the carotenoid contents, including lutein, β-carotene, neoxanthin, Violaxanthin and total carotenoids, in the leaves of three tomato genotypes by HPLC and UV absorption spectroscopy by individual peak areas with similar spectra and retention times revealed an overall increase of the levels of carotenoids in transgenic leaves compared to WT (Table 3.2).

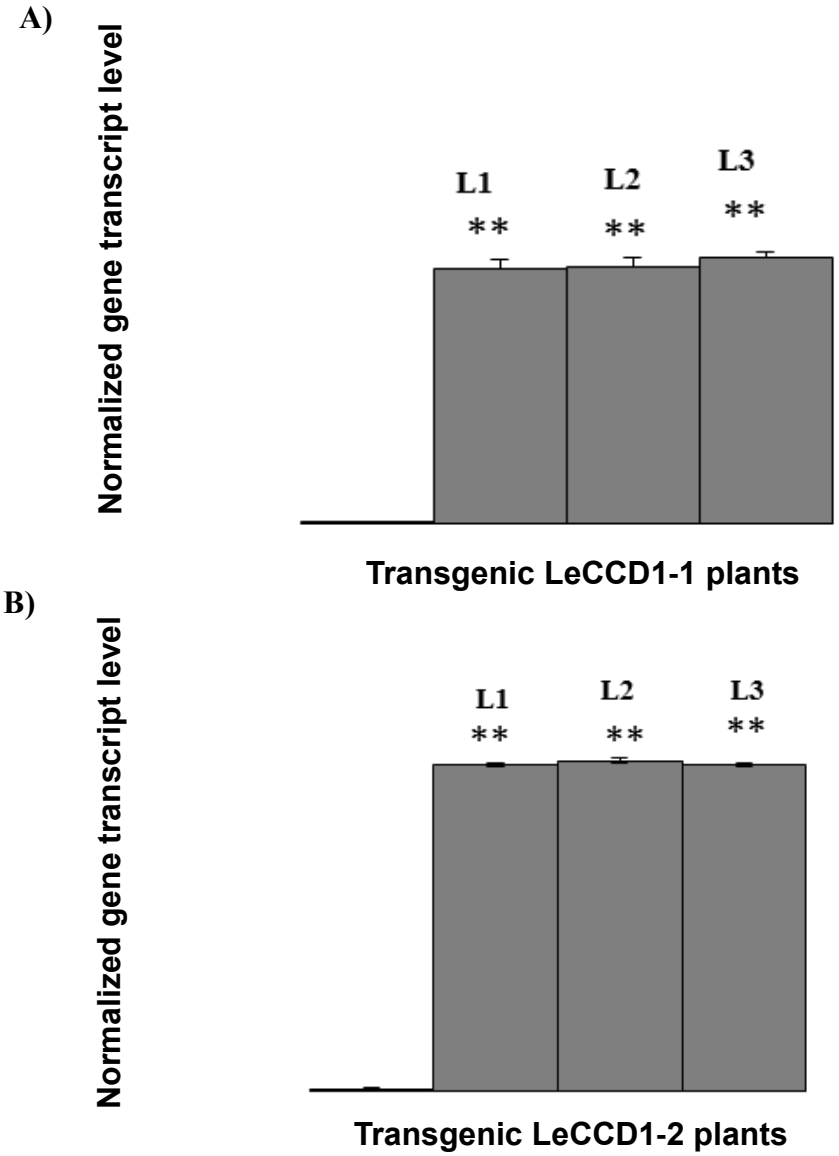

Figure 3.1 Expression profiles of *LeCCD1-1* and *LeCCD1-2* in tomato plants. The level of *LeCCD1-1* transcripts in leaves of four week old transgenic seedlings (A) and the level of *LeCCD1-2* transcripts in leaves of four week old transgenic seedlings (B). Asterisks indicate average ± SE (n=3) are significantly different from non-transformed WT control plants at P<0.005 (*) or P<0.001(**) using one way ANOVA test. L1, L2 and L3 represent individual transgenic lines.

Figure 3.2 Phenotypic comparisons of CCD transgenic tomato plants and WT. No morphological differences were observed between the three genotypes at ten weeks.

A significant increase of violaxanthin, lutein, and β-carotene was detected in all transgenic plants. A comparison of the neoxanthin levels showed that this carotenoid was remarkably constant ($P>0.05$) and only increased by a factor of 1.1. These changes are proportional in that they entail a significant alteration of the total leaf carotenoid contents.

3.3.3 Chemical analysis of tomato headspace volatiles

Twenty two volatile compounds were identified in the headspace of WT and transgenic MicroTom tomato plants (Table 3.3). Terpenoid compounds largely dominated the tomato leaf headspace with α-copaene and R-α-pinene being the most abundant compounds representing more than 50% of the total volatile emission. In addition, cyclosativene, β -pinene, 3-Carene as well as β-caryophyllene were also major compounds in the tomato headspace of the three genotypes.

Although most volatiles were released by all 3 genotypes, the headspace composition differed between transgenic and WT genotypes. The most prominent changes observed were that of α-copaene and β-pinene. A decrease in eucalyptol levels was observed in LeCCD1-2 plants while LeCCD1-1 plants showed a decrease in α-fenchene. . Furthermore, a few other compounds that showed a 1-fold difference in the volatile levels are: β-caryophyllene, δ-elemene, β-phellandrene and sabinene (Table 3.3).

The *AtCCD1* gene in Arabidopsis is known for its role in cleaving the carotenoid molecule to form β-ionone. Considering the similarity of the *CCD1* Arabidopsis gene (Simkin, 2004) to the tomato *CCD1* genes, it was expected to observe a similar function of the gene in the production of β-ionone. However, results from the volatile study (Table 3.3) shows that no β-ionone was detected in the headspace volatiles of tomato plants.

Table 3.2 Effect of overexpression of *LeCCD1-1* and *LeCCD1-2* genes on leaf carotenoids in tomato

Genotype	Line	Lutein	β-Carotene	Violaxanthin	Neoxanthin	Total carotenoids
WT	WT	254 ± 7.0	32 ± 6.5	56 ± 2.6	50.7 ± 16.3	466 ± 38.4**
LeCCD1-1	L1	1129 ± 18.7**	1154 ± 48.5**	145 ± 22.5	53.3 ± 3.5	2900 ± 50.9**
	L2	1265 ± 20**	1148 ± 34.0**	241 ± 27.8	70.6 ± 15.7	2579 ± 18.4**
	L3	1140 ± 21.5**	1138 ± 19.7**	255 ± 25.7	57 ± 10.5	2678 ± 85.0**
LeCCD1-2	L1	1240 ± 19.9**	1367 ± 13.0**	378 ± 17.9*	83 ± 15.9	2853 ± 97.8**
	L2	1261 ± 30.8**	1251 ± 14.5**	360 ± 4.0*	85 ± 7.8	2523 ± 47.1**
	L3	1377 ± 20.9**	1293 ± 52.8**	370 ± 22.1*	91 ± 4.5	2686 ± 56.8**

Levels of Lutein, β-carotene, Violaxanthin, Neoxanthin and total carotenoids in WT and different transgenic genotypes are shown in the table 3.2. L1, L2 and L3 represent the different transgenic lines and values are the average of 3 replicates ± standard deviation.

Comparing the LeCCD1-1 transgenic plants to LeCCD1-2 transgenic plants the overall trend observed is a general decrease in the concentration of most compounds in LeCCD1-2 plants (Table 3.3). Monoterpene Limonene and Sesquiterpenes α-copaene and β-caryophyllene show an increase in the LeCCD1-2 plants when compared to LeCCD1-1 plants. The most significant change is observed in the monoterpene, eucalyptol concentration. Eucalyptol shows a significant decrease in LeCCD1-2 plants in comparison to LeCCD1-1 plants.

3.3.4 Oviposition preference

In the dual choice bioassays the females of both species oviposited more eggs on transformed LeCCD1-1 plants compared to the non-transformed plants (Table 3.4-3.7). The opposite effect was observed during choice assays between LeCCD1-2 and WT plants with the *T. ni* moths (Table 3.4). The use of an oviposition preference index (OPI) allowed for the transformation of a categorical variable into a quantitative one that was analyzed using an analysis of variance (ANOVA) approach. Significantly more eggs were oviposited on the WT control plants by *T. ni* females, while the *T. vaporariorum* females preferred to deposit eggs on the transgenic LeCCD1-2 plants. The proportion of eggs on the transgenic plants relative to WT is highly significant, thereby proving that the insects do distinguish between the different genotypes. In no choice tests, no significant effect on oviposition was noted as approximately equal number of eggs were found on both the genotypes (Table 3.5-3.7).

Table 3.3 Volatile profiles of WT and LeCCD tomato plants

		WT			*LeCCD1-1*			*LeCCD1-2*		
	Compounds	**Average**	**±**	**S.D.**	**Average**	**±**	**S.D.**	**Average**	**±**	**S.D.**
1	O-Xylene	tr	±	0.03	0.07	±	0.04	tr	±	0.01
2	Tricyclene	0.28	±	0.01	0.34*	±	0.1	0.25	-	-
3	α-Pinene	0.27	±	0.04	0.32	±	0.01	0.11**	±	0.02
4	R-α-Pinene	32.16	±	0.03	39.34*	±	0.06	36.21**	±	0.01
5	α-Fenchene	0.52	±	0.03	0.05*	±	0.03	0.35	±	0.04
6	Camphene	0.36	±	0.28	0.35	±	0.25	0.37	±	0.11
7	Sabinene	0.25	±	0.32	0.44*	±	0.33	0.41*	±	0.36
8	β-Pinene	2.05	±	0.08	5.31**	±	0.16	2.86**	±	0.16
9	3-Carene	1.18	±	0.05	1.2	±	0.04	1.17	±	0.07
10	O-Cymene	0.85	±	0.61	0.94*	±	0.78	0.83	±	1.06
11	Limonene	0.79	±	0.71	0.82	±	0.46	0.85*	±	0.69
12	β-Phellandrene	0.82	±	0.03	0.94*	±	0.04	0.54*	±	0.06
13	Eucalyptol	0.31	±	0.04	0.46	±	0.04	0.04**	±	0.07
14	Γ-Terpinene	0.05	±	0.02	1.25	±	0.02	0.1	±	0.06
15	α-Pinene oxide	0.25	±	0.06	0.28	±	0.2	0.19*	±	0.25
16	δ-Elemene	0.56	±	0.52	0.65*	±	0.39	0.68**	±	0.77
17	Cyclosativene	1.54	±	0.06	1.39	±	0.95	1.28**	±	0.07
18	α-Copaene	14.37	±	0.01	7.71**	±	0.08	10.21**	±	0.04
19	Sativene	0.34	±	0.05	0.38*	±	0.06	0.31	±	0.02
20	β-Caryophyllene	5.34	±	0.04	4.12*	±	0.2	5.9	±	0.19
21	Humulene	0.21	±	0.04	0.09*	±	0.05	0.17	±	0.04
22	Caryophyllene oxide	0.4	±	0.03	0.3	±	0.07	0.29	±	0.04

Average ± S.D. represents the relative peak area for each compound and is an average of at least 3 biological replicates. Asterisks indicate average ± S.D. are significantly different from WT control plants at $P<0.05$(*) or $P<0.01$(**) using one way ANOVA. tr indicates trace values.

Table 3.4 Total number of eggs oviposited by *T.vaporariorum* females on WT and transgenic tomato plants in a dual choice assay

Gene	Genotype	Eggs laid				Average ± S.D.	OPI
		R1	R2	R3	R4		
LeCCD1-1	WT	2	4	9	4	19 ± 3	-0.63
	L1	9	16	20	41	86 ± 14	
	WT	15	21	10	15	61 ± 24	-0.57
	L2	51	54	32	90	227 ± 8	
	WT	10	32	45	28	115 ± 17	-0.35
	L3	31	85	65	55	236 ± 16	
LeCCD1-2	WT	12	1	15	5	33 ± 6	-0.67
	L1	42	52	42	29	165 ± 9	
	WT	39	26	19	36	120 ± 9	-0.24
	L2	52	36	35	70	193 ± 16	
	WT	16	19	29	24	88 ± 5	-0.34
	L3	31	37	55	56	179 ± 12	

R1, R2, R3, R4, R5 and R6 represent the 6 replicates used in this study. L1, L2 and L3 represent the different transgenic lines for each genotype. Values are the number of eggs laid on each plant. OPI is oviposition preference index. Negative values and values closer to zero, indicate a preference towards transgenic plants.

Table 3.5 Total number of eggs oviposited by *T. ni* females on WT and transgenic tomato plants in a dual choice assay

Gene	Genotype	Eggs laid				Average ± S.D.	OPI
		R1	R2	R3	R4		
LeCCD1-1	WT	215	164	219	254	218 ± 67	-0.36
	L1	396	365	456	541	462 ± 96	
	WT	252	241	101	415	226 ± 111	-0.30
	L2	512	524	321	390	416 ± 88	
	WT	101	342	345	298	292 ± 97	-0.34
	L3	312	685	564	525	595 ± 167	
LeCCD1-2	WT	422	522	428	529	450 ± 60	0.38
	L1	123	201	152	295	200 ± 64	
	WT	528	348	352	703	476 ± 133	0.26
	L2	396	246	191	326	277 ± 76	
	WT	316	367	557	556	432 ± 106	0.34
	L3	164	149	279	224	210 ± 47	

R1, R2, R3, R4, R5 and R6 represent the 6 replicates used in this study. L1, L2 and L3 represent the different transgenic lines for each genotype. Values are the number of eggs laid on each plant. OPI is oviposition preference index. Negative values and values closer to zero, indicate a preference towards transgenic plants.

Table 3.6 Total number of eggs oviposited by *T. vaporariorum* females on WT and transgenic tomato plants in a no choice assay

Gene	Genotype	Eggs laid				Average ± S.D.	OPI
		R1	R2	R3	R4		
LeCCD1-1	WT	4	2	15	8	29 ± 6	0.07
	WT	6	2	12	5	25 ± 4	
	L1	14	16	30	16	76 ± 7	0.12
	L1	16	15	18	10	59 ± 3	
	L2	19	15	15	16	65 ± 4	0.02
	L2	15	14	17	15	62 ± 4	
LeCCD1-2	L3	16	13	17	10	56 ± 1	0.03
	L3	12	15	12	14	53 ± 1	
	L1	26	19	10	11	66 ± 6	-0.02
	L1	21	17	11	20	69 ± 4	
	L2	31	12	16	14	73 ± 8	-0.05
	L2	35	14	15	16	80 ± 1	

R1, R2, R3, R4, R5 and R6 represent the 6 replicates used in this study. L1, L2 and L3 represent the different transgenic lines for each genotype. Values are the number of eggs laid on each plant. OPI is oviposition preference index. Negative values and values closer to zero, indicate a preference towards transgenic plants.

Table 3.7 Total number of eggs oviposited by *T. ni* females on WT and transgenic tomato plants in a no choice assay

Gene	Genotype	Eggs laid				Average ± S.D.	OPI
		R1	R2	R3	R4		
	WT	145	205	115	162	627 ± 37	0.0
	WT	152	201	124	150	627 ± 32	
LeCCD1-1	L1	214	216	230	262	922 ± 22	0.01
	L1	216	215	218	250	899 ± 16	
	L2	129	125	125	106	485 ± 10	0.0
	L2	125	134	117	105	481 ± 12	
LeCCD1-2	L3	162	163	170	100	595 ± 32	-0.01
	L3	177	155	162	114	608 ± 27	
	L1	262	119	120	212	713 ± 70	0.0
	L1	271	117	111	204	703 ± 76	
	L2	341	122	156	142	761 ± 38	-0.01
	L2	350	124	151	140	765 ± 31	

R1, R2, R3, R4, R5 and R6 represent the 6 replicates used in this study. L1, L2 and L3 represent the different transgenic lines for each genotype. Values are the number of eggs laid on each plant. OPI is oviposition preference index. Negative values and values closer to zero, indicate a preference towards transgenic plants.

3.4 Discussion

In this study, 2 sets of transgenic tomato plants, one over-expressing *LeCCD1-1* gene and the other *LeCCD1-2*, were generated as a means of enhancing *in vivo* VOC emission and allowing for the testing of oviposition preference by *T. ni* and *T. vaporariorum*. The VOCs identified in this study were consistent with those previously described in tomato leaf aroma (Buttery et al., 1987). The total volatile terpene emissions produced constitutively by transformed tomato plants were significantly higher than those detected in the WT plants (Table 3.2).

The volatile profile of tomato plants of the genotypes LeCCD1-1 and LeCCD1-2 was dominated by monoterpenes, in particular R-α-pinene and β-pinene, and the sesquiterpenes (E)-β-caryophyllene and α-copaene (Table 3.2), in accordance with another study (Shu et al., 2010). Nonetheless, overall headspace composition of the transformed genotypes differed significantly from the WT genotype, due to differences in blend proportions of minor compounds and due to the absence of a few compounds detected only in WT tomato headspace.

β-caryophyllene, identified as one of the most abundant sesquiterpene in the headspace of tomato plants has been associated with greenhouse tomato plants under herbivore attack (Miresmailli et al., 2010). Similarly, whitefly oviposition bioassays with commercially available monoterpenes, R-α-pinene and β-pinene, showed these compounds increased the preference for treated leaves as opposed to untreated leaves (Cáceres, 2015) confirming the behavior of the insects can be attributed to the differences in volatiles recorded in the present study.

The differences in volatile profile observed were associated with the over-expression of key carotenoid catabolic genes, *LeCCD1-1* and *Le-CCD1-2*. The *CCD1* gene controls the enzyme that cleaves carotene and the production of β-ionone, the apocarotenoid having insect feeding deterrent activity (Wei et al., 2011). It was determined in the present study that *CCD1* also

affects the production and accumulation of key carotenoids, namely lutein, β-carotene and violaxanthin in the leaves of the LeCCD overexpression plants. Initially, it was predicted that there would be a decrease in the levels of these compounds due to the increase in carotenoid catabolism. This accumulation could possibly be due to a positive feedback regulation in the carotenoid biosynthesis pathway (Verpoorte & Memelink, 2002). The levels of the total carotenoids however remained constant in both the plant genotypes.

The combined headspace analysis, carotenoid composition and behavioural assays indicate that tomato leaf volatile profiles influence host finding and oviposition for both *T. ni* and *T. vaporariorum* (Table 3.2-3.7). Females detected small variations in volatile signatures of the different tomato genotypes that resulted in the observed behavioural response (Table 3.4 and 3.5). The adult *T.ni* females were attracted to plants over-expressing the carotenoid genes Le*CCD1-1* and *LeCCD1-2*, whereas female *T.vaporariorum* showed a preference only for the LeCCD1-1 plants in dual choice assays. These preferences were observed even when the testing arena was very small with a porous gauze opening on the top wall of the chamber. When the headspace volatile profiles did not differ, as in the case of the no choice assay, the insects do not discriminate a difference and oviposition was similar on both plants, regardless if they were transformed or non-transformed (Table 3.6 and 3.7). The differences in the behaviour of the *T.ni* and *T.vaporariorum* females towards the transgenic LeCCD1-2 plants can be due to two reasons: 1) Different species of insects may react differently to the same compound or blend of compounds 2) Differences in nucleotide and protein similarity between the two genes can account for the differences in the insects response to the plant. The two tomato *CCD1* genes showed a nucleotide sequence similarity of up to 83% which is the primary reason for the classification of the two genes into *LeCCD1-1* and *LeCCD1-2* (Simkin et al., 2004). The

predicted tomato proteins however show only 40% similarity to each other. These results are supported by Schwartz et al., 2004 whose study shows a higher similarity of the tomato CCD1 proteins to the Arabidopsis CCD1 protein (Schwartz et al., 2001) and a relatively lower similarity between LeCCD1-1 and LeCCD1-2 proteins. These predicted differences could better explain the oviposition choices of *T.vaporariorum* females.

No obvious difference in trichome density between the leaves of the WT and the transgenic plants were observed making it unlikely to be the cause of the higher emission of VOCs by the transgenic plans. This is in contrast to the finding (Li et al., 2012) which correlated the reduced constitutive VOC emissions from mutant tomato with lower trichome density. Another study found that trichome density was directly correlated with spider mites *Tetranychus urticae* (Trombidiforme: Tetranychidae) deterrent activity on the tomato leaf surface (Maluf et al., 2007). Higher densities decreased the walking distance of the spider mites which was measured as an index of mite repellence. The present results, on the other hand, showed a significant difference in the oviposition choice but no obvious difference in trichome densities between WT and transformed tomato. This further confirms that the change in insect behaviour observed was not a result of trichome interference.

For most insect species, olfactory cues provide information to locate and identify appropriate host plants to oviposit their eggs, including the predation risk associated with them. Repellance responses towards plants are involved with volatile emissions, as has been recorded for several moth species, including *Manduca sexta* (Heath et al., 1993). In contrast, the significantly higher volatile emissions produced by the transgenic plants appeared to have a stimulatory effect on the fecundity of *T. ni* and *T. vaporariorum*. One explanation for the oviposition preference by *T. ni* and *T. vaporariorum* females for transgenic plants could be the necessity to increase the chances

of offspring survival. Having detected the transgenic plant as a potentially less favorable host for larval development, these insects may have increased their egg load in order to ensure maximum larval survival rate. This explanation is in context with "mother-knows-best" hypothesis also termed as "preference-hypothesis" (Clark et al., 2011; Jaenike, 1978; Valladares & Lawton, 1991). However, further studies with commercially available compounds would help to better understand the response of these insects to different compounds.

The ultimate goal of this study is to manipulate the genetics of the plants as an alternative strategy for pest management and plant protection thereby reducing reliance on chemical pesticides. These results, although seemingly counter-intuitive to protecting the plant by repelling pests, can still be applied for crop protection within a "push-pull" strategy. In this case the "push" can come from an anti-feedant or deterrent effect from both a crop or non-host plant, while the "pull" can come from an attractant effect from a non-host plant acting as a "trap crop". A number of terpenoids produced by the transformed plants could cause a "behavioral manipulation", attracting a mobile adult insect to abandon an otherwise suitable host plant some distance away. In this way the main crop will be protected.

Furthermore, the fact that some of these terpenoid volatile compounds come from natural sources may present useful alternatives to commercially available synthetic insecticides in the market. In addition, the fact that no new gene was introduced into the plant (native endogenous gene from tomato was overexpressed in tomato), gives the transgenic plant an edge over the traditional genetically modified crops such as Bt cotton (gene coding for Bt toxin was introduced into cotton plant from the bacterium *Bacillus thuringiensis*). These results also contribute to the abundant literature showing that plant volatiles influence oviposition behavior. Evidence of the ability of herbivores to use chemical cues of transformed plants to locate food and suitable

oviposition sites is exciting because if proven useful in large scale field studies, this strategy can further reduce the use of insecticides and help protect the environment and other non-target organisms from the negative impacts of chemicals. This further also creates an incentive for plant breeding to enhance the genetic trait underlying the volatile emissions from plants and in such a way maximize the impact of natural plant volatiles in the biological control of insect pests.

In conclusion, these results indicate that terpenoids compounds are responsible for the attraction of *T. ni* and *T. vaporariorum*, and could be used to protect plants in the greenhouse or field as part of a "push-pull" strategy.

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Chapter 4. Conclusion

This thesis reported the influence of volatile compounds derived from transgenic Arabidopsis over-expressing the *CCD1* and *CCD4* genes on cabbage looper moth oviposition preference (Chapter 2) and the molecular and physiological aspects of transgenic tomato over-expressing *LeCCD1-1* and *LeCCD1-2* genes and the response of both the cabbage looper moths and the greenhouse whiteflies to the transformed plants (Chapter 3).

The results of the studies reported in Chapters 2 and 3 indicate that the oviposition preference of these two insects was affected by the genotype of the plant. These studies include molecular characterization of the plants, investigating the carotenoid composition of the different genotypes for each model plant, identifying the volatile constituents of the genetically transformed and wild-type plants and determining the effects of carotenoid-derived volatiles on the feeding and oviposition choices made by the two insects.

4.1 Preferential foraging and plant genetic makeup

When the plant genetic makeup was kept constant in a closed chamber no choice experiment, the insects did not display a bias towards either of the two plants in the chamber and a similar pattern of feeding and oviposition was observed for both plants. However, when the insects were given a choice between different plant genotypes, an oviposition preference towards the transgenic plants was observed in most cases except for the LeCCD1-2 tomato-cabbage looper model system (Chapter 3). The cabbage looper moth showed a preference to the LeCCD1-1 tomato plant and CCD1 and CCD4 Arabidopsis plants compared to the WT untransformed plants, an observation that was consistent with the greenhouse whitefly response. Only the LeCCD1-2 tomato plants were more attractive to the cabbage looper moths over the WT tomato plants. The greenhouse

whiteflies consistently preferred the transgenic plants from both tomato and Arabidopsis species. This discrepancy in the trend of oviposition by cabbage looper moth can be partially explained by the fact that tomatoes do not belong to Brassicaecae, the preferred host plant family of cabbage looper. A more substantial finding from the data in Table 3.2 is that there are significant differences in the composition of a few compounds between LeCCD1-1 and LeCCD1-2 plants. In general, a decrease in concentration of compounds was observed in LeCCD1-2 plants in comparison to LeCCD1-1 plants. The monoterpene eucalyptol stands out the most with the LeCCD1-2 plants showing the most significant decrease in concentration. The difference in the levels of these compounds was also observed in a similar study conducted by Cáceres, 2015. Thus, the observed differences in the oviposition behavior of the moths can be attributed individually to one of these compounds or to a blend of volatiles, pending further confirmation with synthetic compounds.

Since plant availability and developmental stage were kept constant and insects were held under controlled laboratory conditions before and during the bioassays, individuals chose the plants based on the differences in their volatile profiles. The differences in volatile profile recorded in Chapter 2 and 3 are due to overexpression of the *CCD* genes in both tomato and Arabidopsis, respectively. *CCD* genes are responsible for cleaving a broad range of carotenoids found in plants, such as lycopene, β-carotene, zeaxanthin, violaxanthin, and neoxanthin to generate aldehydes and ketones that are volatile aroma compounds (Auldridge et al., 2006; Wei et al., 2011). In tomato fruits, for instance, *CCD* generates flavour volatiles such as geranylacetone, pseudoionone and β-ionone (Simkin et al., 2004). The apocarotenoid volatiles are produced by the cleavage action of the carotenoid cleavage dioxygenase genes and include: β-ionone, α-ionone, 3-hydroxyl- β-ionone and geranylacetone.

As described in Chapters 2 and 3, the transgenic tomato and Arabidopsis plants were genetically transformed to overexpress two endogenous *CCD* genes. The two genes, *CCD1* and *CCD4*, were overexpressed in the Arabidopsis plants while *LeCCD1-1* and *LeCCD1-2* genes were over-expressed in tomato plants. The overexpression of these genes led to changes in the carotenoid profiles and the transcript levels of a few structural genes from the carotenoid biosynthetic pathway. All these factors combined together altered the genetic makeup and volatile profiles of the plants thus influencing the response of cabbage looper moths and greenhouse whiteflies.

4.2 Volatiles as important plant pest control metabolites

It is clear from previous studies that volatiles are an important defense strategy employed by most plants, significantly influencing feeding and oviposition choices of insects above and below ground. Research on terpenoid-derived volatiles has been gaining momentum in the past few years. This is mainly due to the increase in commercial demand for a safe and environmentally friendly pest management strategy. Similarly, the dietary importance of carotenoids and the range of diverse biological functions and actions attributed to carotenoids are factors responsible for the enormous growth of interest in the carotenoid biosynthesis pathway. However, the effect of altering these genes on the carotenoid content and subsequently on the volatile profile of the plants is largely unknown. Previously, a wide variety of volatiles were reported from transgenic tomato and Arabidopsis plants overexpressing *CCD* genes (Cáceres, 2015; Lakshminarayan, 2013; Wei et al., 2011), but the role of these volatiles in plant-insect interactions had not been thoroughly investigated. The focus of my thesis was to study CCD over-expressing plants in order to isolate and identify the volatile apocarotenoids and to further investigate insect response

to these volatiles. Despite interesting and promising results, the study is extremely complicated and could legitimately form the basis of a PhD project on its own.

Further experimental testing using individual synthetic compounds that were identified could help isolate the key volatile compounds responsible for the insect attraction. Studies on the effect of other *CCD* genes apart from the ones investigated in this experiment would also contribute to a better understanding of the mechanisms controlling the production of different apocarotenoids or terpenes. Clearly, more research is required to identify and quantify the biologically active volatile compounds in different plant species and genotypes, and characterize the ecological interactions between these plants and insects.

4.3 Prospects for future research

The results of my oviposition preference experiments clearly show that altered volatile profiles influenced cabbage looper moths and whiteflies, and have a potential for application as a pest management strategy. While the insects distinguished between the two genotypes in a closed chamber, it would be interesting to investigate the extent of this effect in a more realistic setting. Would the insects be able to detect the volatiles from a greater distance or when a greater number of plants are present? A different experimental design would be required with multiple plants to elucidate whether preferential oviposition occurs.

Since the results for the cabbage looper larval feeding trials conducted as a part of this project showed no differences between either genotype (i.e., the transgenic and WT genotypes), future questions could be: 1) does larval development stage influence the preference for one genotype over another and 2) would larval performance be different on the transgenic versus WT plants thus indicating the suitability of the transgenic plants as hosts? As was discussed earlier in

Chapters 2 and 3, the nutritional status of the plants can also be a factor influencing the adult female moth. Follow up experiments could examine the dry weight (relative growth rate) of the larvae fed transgenic plants for a longer period of time than the 24 h period used in the present study. This experiment could shed more light on the quality of the plant as a food source for the developing larvae.

The concept of this research was based on the initial study by Wei et al. (2011) on the deterrent effects of overexpressing *CCD1* in Arabidopsis on crucifer flea beetle herbivory. However, the feeding trial results from my thesis conducted with cabbage looper larvae indicate no feeding deterrent effect with the different CCD genotypes (i.e., *CCD1*, *CCD4*, *LeCCD1-1*, *LeCCD1-2* and WT) indicating that the effect of volatiles might be species-specific. Hence, it would be interesting to look at other species of insects in order to elucidate the effect of these volatiles on feeding and oviposition choices. This would assist the development of recommendations as to whether the transgenic plants used in this project would be useful for insect pest management and crop protection programs. Certainly the safer and improved environment health offered by these plants is an incentive to be studied further.

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